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ABSTRACT

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2004

Major Advisor: Patricia Guerry, Ph.D., Department of Enteric Diseases, Naval Medical Research Center

Title of Dissertation: Characterization of a plasmid-encoded type IV secretion system in *Campylobacter jejuni* 81-176.

The Gram-negative bacterium *Campylobacter jejuni* is major cause of diarrheal illness in both the United States and the world abroad. Although well established as an etiological agent, little is understood regarding the molecular mechanisms underlying its pathogenesis. Additionally, inter-strain variations in clinical manifestations, *in vitro* epithelial cell invasion levels, and virulence in animal models suggest that genetic elements account for these phenotypic differences.

One strain of *C. jejuni*, 81-176, is a particularly virulent strain that invades epithelial cells at levels higher than most *C. jejuni* isolates. This strain possesses two

plasmids, designated pVir and pTet, both of which contain genes with homology to type IV secretion systems (TFSS). TFSS are systems capable of translocating DNA, protein, or nucleoprotein complexes across bacterial membranes. Such systems are found in a number of human pathogens, where they facilitate various pathogenic processes. A mutational analysis in some of the pVir TFSS genes reduced levels of intestinal epithelial cell invasion and natural competence. Additionally, a mutation in one of the TFSS genes led to an attenuation of virulence in the ferret diarrhea model. These data led us to hypothesize that the pVir TFSS contributes to the pathogenesis of *C. jejuni* 81-176 and accounts for the phenotypic differences observed in virulence models.

This work represents a characterization of the pVir TFSS found in *C. jejuni* 81-176. Following purification and the generation of antisera, it was demonstrated that VirB10, a structural component of the pVir TFSS is glycosylated. Lectin affinity, enzymatic cleavage, and a reconstitution of glycosylation in *Escherichia coli* suggested that VirB10 was glycosylated by the general *N*-linked glycosylation pathway (*pgl*) of *C. jejuni.* Site-specific mutagenesis of VirB10 revealed two glycosylation sites at N32 and N97. Previously, mutation of *virB10* resulted in a modest reduction in levels of natural competence. Mutation of N97 but not N32 resulted in levels of natural competence consistent with the *virB10* mutant, suggesting glycosylation is required for the function of the pVir TFSS.

Secondly, an initial biochemical characterization of a putative type IV secretion ATPase was undertaken. A VirB11 homolog from the pVir plasmid was expressed and purified. This protein was shown in both a time and concentration dependent

manner to possess ATPase activity and mutation of the nucleotide-binding site of VirB11 resulted in a form of the protein that was devoid of enzymatic activity. Yeast 2-hybrid analysis and chemical cross-linking experiments suggested that VirB11 formed hexameric structures, consistent with other VirB11 family members.

Lastly, characterization of a pVir-encoded protein linked with the TFSS was performed. This protein, Cjp29, bears homology to eukaryotic cell proteins and its expression is dependent on the TFSS genes. The regulation with the TFSS suggests that Cjp29 may be a protein substrate of the pVir TFSS.

Taken together, the characterization of the pVir TFSS represented here has provided a substantial foundation upon which to launch future research to elucidate the specific contributions of this system to clinical illness.

Characterization of a plasmid-encoded type IV secretion system in *Campylobacter jejuni* 81-176

by

Joseph C. Larsen

Thesis/dissertation submitted to the Faculty of the Emerging Infectious Diseases Graduate Program Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy 2004

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CHAPTER 1

The Virulence Plasmid of *C. jejuni* 81-176

Introduction

Despite years of research and availability of a complete genome sequence (Parkhill et al., 2000), there is remarkably little understood about the molecular pathogenesis of *C. jejuni*. While several individual virulence determinants have been extensively characterized, most notably flagella and the cytolethal distending toxin, our understanding of the overall disease process remains rudimentary. The genome sequence revealed the presence of genes encoding a previously unknown capsule, which has subsequently been shown to be the serodeterminant of the Penner serotyping scheme and essential for intestinal cell invasion (Karlyshev et al., 2000; Bacon et al., 2001). However, the genome sequence failed to reveal any specific secretion systems, type III or type IV, that form the basic framework for many other bacterial pathogenesis schemes. The disease caused by *C. jejuni* is manifested by a range of clinical presentations, from a mild watery diarrhea to a dysentery syndrome. These differences could be due to either variation in host response to infection or to differences in virulence among individual strains, or a combination of the two. However, it is clear that there are major differences among strains in their ability to invade intestinal epithelial cells *in vitro*. Our laboratory has focused on one strain of *C. jejuni*, 81-176, which was originally isolated during an outbreak of diarrhea in children who consumed raw milk during a visit to a dairy farm (Korlath et al., 1985). The selection of this strain was based on the fact that 81-176 was fed to human volunteers on two occasions over 28 years apart and caused disease in both instances (Black et al., 1988; Tribble, unpublished), and that the strain invades intestinal epithelial cells at much higher levels than most other strains (Oelschlaeger et al., 1993). In contrast, the genome strain,

NCTC 11168 invaded INT407 cells at <1% the level of 81-176 (Bacon et al., 2000). Moreover, 81-176 causes disease in the ferret diarrhea model, but NCTC11168 is avirulent (Bacon et al., 2000). These significant differences in virulence phenotypes must be due to genetic determinants.

Microarray hybridizations have indicated that about 5% of the genome of NCTC 11168 is missing in 81-176 (Dorrell et al., 2001). Comparative genomic analyses in our laboratory have confirmed that a number of 11168 genes are deleted in 81-176 and we have identified a limited number of novel chromosomal genes (Guerry, in preparation). The large proportion of differences between the strains lies within loci encoding for the LOS and capsule (Guerry, in preparation). Subtracting these differences, one finds only eleven unique genes in 81-176. The most obvious genomic difference between 11168 and 81-176, however, is the presence of two large plasmids in the latter strain.

Various studies have shown that a significant proportion of *C. jejuni* strains harbor plasmids. Reports have indicated that anywhere from 19 to 53% of strains contain plasmids (Austen et al., 1980; Taylor et al., 1981, 1983; Bradbury et al., 1983, 1985; Bopp et al., 1985; Sagara et al. 1987). While a majority of plasmids harbored by *C. jejuni* and *C. coli* strains contain genes encoding antibiotic resistance, there are reports of cryptic plasmids (Bopp et al., 1985).

One of the two plasmids found in *C. jejuni* 81-176, pTet, is a conjugative R factor encoding tetracycline resistance (Batchelor et al., 2004), and is likely related to the *tetO* plasmids previously described by Taylor et al. (Taylor et al., 1983). The pTet plasmid is 45 kb in size and transfers to other strains of *C. jejuni* at frequencies from

10^{-4}-10^{-6} per donor cell (Batchelor et al., 2004). Conjugative transfer of pTet is mediated by a type IV secretion system encoded on this plasmid (see below), similar to those found on conjugative plasmids from other bacterial genera. Plasmids related to pTet are ubiquitous in *C. jejuni* isolates from Thailand that display multiple drug resistance (Warawadee, Mason, and Guerry, in preparation). Moreover, loss of pTet had no effect on invasion of intestinal epithelial cells (Bacon et al., 2000). The second plasmid found in 81-176, designated pVir, is non-conjugative and has been implicated in virulence. This plasmid characteristics, prevalence, and potential involvement in *C. jejuni* pathogenesis will be reviewed in depth throughout this chapter.

General Characteristics of pVir

The pVir plasmid of *C. jejuni* 81-176 is 37,468 nucleotides in length (Bacon et al., 2002). A circular map of pVir is shown in Figure 1. The G+C content of the plasmid is 26%, which is significantly lower than that of the published *C. jejuni* NCTC11168 chromosome (Parkhill et al., 2000), suggesting that the plasmid may have been acquired by horizontal transfer. The G+C content of pVir was also lower than a number of other previously identified *C. jejuni* plasmids (Luo and Zhang, 2001), with the exception of a plasmid from *Campylobacter hyointestinalis* (Waterman et al., 1993). The pVir plasmid contains 54 predicted ORFs, designated Cjp for "*Campylobacter jejuni* plasmid". The plasmid has very few intergenic regions, devoting 83% of the DNA as coding sequence, which is also similar to the coding density of the NCTC11168 chromosome (Parkhill et al., 2000). Two intergenic non-coding regions exist within pVir, one of 1,147-bp present between *cjp28* and *cjp29*. The other non-coding region of 1,049-bp exists between *cjp49*

and *cjp50*. The intergenic region between *cjp28* and *cj29* contains a repetitive region that is 260-bp in length and is flanked by 52-bp direct repeats as well as by 36-bp inverted repeats. These elements may serve as an origin of replication due to minor similarities with the origin of small cryptic plasmid identified in some strains of *Campylobacter* (Bacon et al., 2002; Luo and Zhang, 2001). Data presented later in this thesis suggests, however, that this region plays a regulatory role in *cjp29* expression. Although repeat regions often bracket transposable elements, the pVir repeats do not contain homology to any known transposable element. Further, transposable elements have not been identified to date in *Campylobacter* (Parkhill et al., 2000).

Prevalence of pVir

Bacon et al. determined that Cjp5, a gene homologus to the TFSS *virB11*, was present on plasmids in 10.3% of isolates obtained from U.S. military personnel in Thailand (Bacon et al., 2000). Although these strains possessed Cjp5/*virB11*, it is unknown if the total repertoire of plasmid genes present in these strains was similar to pVir. In the same study, a variety of laboratory strains were also screened and these strains lacked the presence of Cjp5/*virB11*. One of these strains, A3249, had caused diarrheal disease in a human challenge study, although the symptoms were much less severe than parallel feeding studies with 81-176 (Black et al., 1988). Recently, a DNA microarray was used to examine various genetic profiles among certain *C. jejuni* strains (Leonard et al., 2003). This study also demonstrated the presence of pVir specific sequences in a significant proportion (31.2%) of strains examined (Leonard et al., 2003). This evidence suggests that pVir or

plasmids of similar genetic content exist in nature in a significant proportion of *C. jejuni* strains.

Contribution of pVir to virulence of *C. jejuni* 81-176

As discussed in depth below, mutation of several genes present on pVir results in reductions in intestinal epithelial cell invasion (Bacon et al., 2000; 2002). One of the non-invasive mutations was in Cjp5/*virB11*, and this mutant is also attenuated in the ferret model of diarrheal disease (Bacon et al., 2000). However, electroporation of pVir into NCTC11168 did not increase *in vitro* invasion levels (Bacon et al., 2002). This suggested that while the plasmid is required for full invasion of 81-176, there are other unique determinants in 81-176 that are responsible for the higher level of invasion when compared to the genome strain NCTC 11168. Strain-specific surface structures present in 81-176, such as capsule and LOS, which have been shown to modulate virulence (Bacon et al. 2001; Guerry et al. 2002), are among the candidates to account for these observations. Nonetheless, there remain numerous unanswered and puzzling questions regarding the role of pVir in virulence of 81-176, as discussed below.

Genes Present on pVir with Homology to Type IV Secretion Systems

Type IV secretion systems (TFSS) are bacterial protein secretion systems, which are ancestrally related to conjugation machines (Christie and Vogel 2000; Christie 2001). These secretion systems have been shown to transfer DNA, protein, or nucleoprotein complexes across bacterial membranes. It is now accepted that there are three main classes of TFSS, examples of which are shown in Figure 2. The first group is involved in the conjugal transfer of genetic material, like the TFSS involved in the transfer of the pTet

plasmid in *C. jejuni* 81-176 (Bachelor et al., 2004). The second class of TFSS is those systems involved in the uptake or release of DNA. The only current example of a DNA uptake system is the *comB* system of *H. pylori*, mutation of which completely abolished competence (Hofreuter et al., 1998, 2001). A system involved in the secretion of chromosomal DNA was identified in *Neisseria gonorrhoeae* (Dillard and Seifert 2001). The last class of TFSS identified to date secretes protein or nucleoprotein complexes either into the extracellular milieu or directly into eukaryotic cells (Cascales and Christie 2003). Many of these TFSS contribute to virulence in a variety of pathogens.

Agrobacterium tumefaciens, which contains the most studied TFSS, uses its system to translocate the oncogenic T-DNA and protein substrates inside plant cells resulting in crown gall disease. Recently, a DNA immunoprecipitation assay was developed that allowed investigators to elucidate the pathway and energetic requirements of T-DNA transfer through the TFSS apparatus (Cascales and Christie 2004 b). They found that the T-DNA makes contact with six of the twelve proteins that make up the TFSS apparatus (Cascales and Christie 2004 b). This assay was also applied to elucidating the various energetic requirements for substrate secretion (Atmakuri et al., 2004). It was shown that the three ATPases present in the A. *tumefaciens* system mediate early steps in the DNA transfer reaction, consistent with the functions as ATPases (Atmakuri et al., 2004). Although much is understood regarding TFSS in *A. tumefaciens*, the TFSS and particularly TFSS substrates in other pathogenic organisms remain ill defined.

Figure 3 shows the genetic organization of TFSS from *C. jejuni* 81-176, *Wolinella succinogenes*, *Helicobacter pylori*, and *Agrobacterium tumefaciens*, and Table 1 presents the homologies between the various type IV secretion system-proteins encoded by

these genes. The TFSS present on pVir shares greatest similarity to a recently identified TFSS in the ruminant commensal *W. succinogenes* (Baar et al., 2003). Additionally, the TFSS on pVir bears significant homology to two of the three TFSS in *H. pylori*. One of these *H. pylori* TFSS, which was recently identified in an archived stock of the second genome strain, J99, is located within the *H. pylori* plasticity zone (see below) and has not yet been assigned a function (Kersulyte et al., 2003). The other related system is the *comB* system found in all strains of *H. pylori*, which has been directly implicated in natural competence (Hofreuter et al., 1998; 2001). Both of these *H. pylori* systems are distinct from the TFSS present on the *cag* pathogenicity island, which is responsible for the translocation of CagA into gastric epithelial cells (Odenbreit et al., 2000). It is noteworthy that the second TFSS in *C. jejuni* 81-176, which is present on the conjugative pTet plasmid appears to be distinct from the pVir encoded TFSS (Batchelor et al., 2004), as shown in Table 1. Although homologs exist between the *comB* system and the major structural components of the *C. jejuni* TFSS, two proteins of the pVir system, Cjp5 and Cjp6, are not encoded by the *comB* system and have homology to proteins present in the *cag* TFSS.

As mentioned above, it was previously demonstrated that the *comB* locus is directly involved in the natural competence of *H. pylori* strains. Orthologs of the basic structural components of the *comB* TFSS are present on pVir and are designated Cjp54, 1, 2, and 3 (Bacon et al., 2000; 2002). Mutational analysis of Cjp2 and Cjp3 resulted in significant reductions in intestinal epithelial cell invasion (Bacon et al., 2000; 2002). As with its homolog in the *comB* system of *H. pylori*, insertional mutation of the *cjp3* gene also resulted in diminished natural competence. However, the magnitude of this reduction was less than that for the *comB3* mutant phenotype. Additionally, strains of *C. jejuni* that lack

pVir are fully competent, suggesting that other chromosomally encoded competence systems exist. These facts, coupled with the recent identification of a type II secretion system which mediates competence in *C. jejuni* 81-176, as well as observations in our laboratory, suggest that the contribution of pVir to natural competence is a moderate one (Wiesner et al., 2003). The competence defect in the Cjp3 mutants, while modest, appears to be genuine, since the defect can be complemented *in trans* (Larsen et al. 2004). Cjp54 is an ortholog of VirB7 present in *A. tumefaciens* (Goodner et al, 2001; Bacon et al., 2002). This protein is an outer membrane lipoprotein that is covalently linked to another TFSS structural component, VirB9 by intermolecular disulfide bonding (Anderson et al., 1996; Spudich et al., 1996). The presence of a putative lipobox and three Cys residues within its short 42 amino acid sequence suggests that Cjp54 probably fulfills a similar role in *C. jejuni*. Cjp1 is a homolog of ComB1 in *H. pylori* and orthologous to VirB8 in *A. tumefaciens*. This protein is proposed to be an inner membrane protein and was shown in *A. tumefaciens* to interact with VirB9 and VirB10 (Das et al., 2000).

Other components of the pVir TFSS are predicted to be localized and presumably function in a similar capacity to other TFS structural components. Cjp2 is a ComB2/VirB9 ortholog, and is predicted to be an outer membrane protein. Cjp3 is an inner membrane protein with a large periplasmic domain and is thought to span the periplasm to interact with Cjp1 and Cjp2, since a similar role was defined for its ortholog, VirB10, in *A. tumefaciens* (Das et al., 2000). Recently, it was determined that *A. tumefaciens* VirB10 is an ATP sensor that undergoes a conformational change in response to the hydrolysis of ATP by two TFSS ATPases, VirD4 and VirB11 (Cascales and Christie 2004 a). Using the DNA immunoprecipitation assay, the investigators demonstrated that VirB10 alters its

protein interactions from the inner membrane components of the TFSS to outer membrane components upon the hydrolysis of ATP (Cascales and Christie 2004 a). The existing model is that VirB10 funtions as an ATP-dependent bridge that links the components of the TFSS.

The other TFSS homologs present on pVir are presumed to provide energy for substrate secretion or assembly of the secretion apparatus (Fullner et al., 1994; Stephens et al., 1995; Dang et al., 1999). Cjp5 shares homology with a family of hexameric ATPases, including *H. pylori* HP0525 and the *A. tumefaciens* VirB11. These proteins are believed to form hexameric rings on the cytoplasmic side of the inner membrane and provide the energy for substrate secretion (Krause et al., 2000). Recently, Savvies et al. proposed a model based on data obtained from crystal structures of HP0525 (Savvies et al., 2003). This model suggests that the homohexameric form of this protein is dynamic and through the hydrolysis of ATP may provide mechanistic force necessary for assembly of the TFSS secreton or substrate secretion (Savvies et al., 2003). Mutational analysis in *A. tumefaciens* demonstrated that a consensus nucleotide-binding site (Walker A box) is required for virulence (Stephens et al., 1995). A similar motif (GTGGSGKT) is seen in Cjp5, with amino acid residues 150-157. Insertional inactivation of *cjp5* resulted in a dramatic decrease in the adherence and invasion of intestinal epithelial cells, to approximately 10% of the wild-type levels for both phenotypes (Bacon et al., 2000). Additionally, the *cjp5* mutant was also attenuated in the ferret model of disease, consistent with the *in vitro* invasion defect (Bacon et al., 2000). These data suggest that Cjp5 may be the ATPase involved in providing energy necessary for TFS secreton assembly or the secretion of effector proteins.

Two other putative TFSS ATPases are encoded by *cjp6* and *cjp53*. Cjp53 shares homology to the *H. pylori* VirB4 protein. In *A. tumefaciens*, this protein is thought to aid in T-pilus assembly (Dang et al., 1999). Other studies have also shown that the ATPase activity of VirB4 was essential for the transfer of substrates (Berger et al., 1994; Fullner et al., 1994). Further, in *H. pylori*, mutation of this gene completely abrogated natural competence (Hofreuter et al., 2001). Recently, a signature tagged mutagenesis analysis of *H. pylori* identified *comB4* as being essential for colonization of gerbils (Kavermann et al., 2003). Natural competence was determined not to be essential for colonization, suggesting that this protein may have alternate roles in virulence. In 81-176, however, mutation of the *virB4* homolog, *cjp53*, has no effect on intestinal epithelial cell invasion (Bacon et al., 2002). It will be necessary to determine if mutation of *cjp53*, like *H. pylori comB4*, will lead to an *in vivo* colonization defect. The studies of Cjp53 orthologs provide a framework to experimentally address the role it plays in the pVir TFSS function and its potential contribution to virulence.

Similarly, Cjp6 is an ortholog of VirD4, and mutation of this gene has no effect on intestinal epithelial cell invasion (Bacon et al., 2002). In *H. pylori*, it was determined that there were VirD4-dependent and independent functions of the TFSS (Selbach et al., 2002). This study demonstrated that secretion of CagA through the TFSS was dependent on VirD4, while the induction of IL-8 was not. Since mutation of *cjp6* had no effect on intestinal epithelial cell invasion, it is possible that there are as yet unknown processes, which are dependent on Cjp6-dependent energy coupling.

In summary, the published data suggest that the TFSS homologs present on pVir comprise a functional secretion channel that is involved in intestinal epithelial cell invasion

and natural competence. However, there are many issues regarding this system that remain enigmatic. First, mutation of *cjp5* has a dramatic effect on INT407 cell invasion and virulence but has no effect on natural competence (Bacon et al., 2000). This would seem to suggest that this system has two or more functions, with some components serving only one or the other pathway. Secondly, the lack of a phenotype associated with mutation of the ATPases (Cjp6, Cjp53) that are essential in other systems is particularly problematic. Further work will be needed to determine the contribution of these ATPases to the function of the TFSS. Lastly, no specific substrate of the TFSS has yet been identified. Studies in *A. tumefaciens* have suggested that the C-terminal end of TFSS substrates is important for export (Simone et al., 2001). Studies have suggested the presence of a conserved RXR motif present in the C-terminal 18 amino acids of VirE2, a substrate of the TFSS in *A. tumefaciens* (Simone et al., 2001). However, the only protein that satisfies these criteria encoded on pVir is Cjp34, a protein of unknown function that is predicted to be localized to the inner membrane. This may suggest that other elements, either structural or compositional, contribute to substrate recognition by the TFSS in *C. jejuni*. Identification of substrates of the TFSS will provide key insights into the molecular mechanisms of invasion and interaction of *C. jejuni* with the eukaryotic cell.

Genes Homologous to *C. jejuni* Chromosomal Genes

Two genes are present on pVir that shared significant homology to genes present on the NCTC11168 chromosome. One of these genes, *cjp32*, shares significant homology to two genes, *cj0041* (21% identity, 40% similarity) and *cj0320* (22% identity, 43% similarity) over the entire length and 209 amino acids of Cjp32, respectively. The *cjp32*

gene encodes a predicted protein of 239 amino acids, which is shorter than the two homologous proteins that consist of 598 and 276 amino acids, respectively. While Cj0041 does not have significant homology to any known protein, mutation of *cj0041* affected both motility and invasion (Golden et al., 2002). Cj0320 is homologous to FliH, a protein involved in flagellin export. In *Salmonella*, FliH interacts with another protein, FliI, and inhibits its ATPase activity, both of which are needed for flagellar export (Claret et al., 2003; Minamino et al., 2000). Mutation of *cjp32* results in a drop in intestinal epithelial cell invasion to 34.8% of wild-type (Bacon et al., 2002). Another pVir gene, *cjp48* has homology to *cj1456c*, a *C. jejuni* gene of unknown function. Mutational analysis of this gene has yet to be performed.

Genes with Homology to DNA Binding/Replication Proteins

Several genes on the pVir plasmid encode proteins with homology to proteins involved in either DNA replication and/or DNA binding (Bacon et al., 2002). It is important, however, to note that mutations in none of these genes affected plasmid replication or segregation, suggesting a divergent role for these proteins (Bacon et al., 2002). Cjp11 possesses homology to DNA topoisomerase I proteins that are involved in DNA replication. The greatest similarity is shared with topoisomerases encoded by the plasticity zones of *H. pylori* (Tomb et al., 1997; Alm et al., 1999; Bacon et al., 2002). The plasticity zone topoisomerases exist in conjunction with their orthologs present elsewhere on the *H. pylori* chromosome, also suggesting that these proteins may also function in a capacity separate from DNA replication. Another pVir-encoded protein, Cjp13, has homology with single-stranded DNA-binding proteins. Two other genes, *cjp26* and *cjp28*

encode predicted proteins with homology to proteins involved in plasmid replication. Cjp26 is homologous to ParA, a protein involved in the partitioning of newly replicated plasmids to daughter cells. A Cjp26 mutant pVir plasmid, however, was stably replicated and maintained suggesting that this gene is not involved in plasmid replication (Bacon et al., 2002). Interestingly, a *cjp26* homolog is found flanking the region of TFSS genes in *W. succinogenes* (Baar et al., 2003). This may suggest its involvement in the transfer and subsequent integration of this genetic element in *Wolinella*. Cjp26 is also a homolog of HP1000, a gene in the plasticity zone of *H. pylori* 26695 (see below). Cjp28 shows homology to another replication protein, RepA. Cjp28 appears to be a pseudogene, however, due to the presence of an in-frame stop codon within the coding sequence.

H. pylori Plasticity Zone Homologs of Unknown Function

In addition to *cjp11* and *cjp26*, there are five other genes present on pVir that are homologous to genes present within the plasticity zone of *H. pylori* J99 or 26695. The sequencing of two different *H. pylori* isolates revealed that the genome organization between the two strains was highly conserved, with the exception of one region present in both strains that was highly variable in its genetic content (Alm et al., 1999). These two isolates had different clinical presentations, with one strain causing gastritis and the other manifesting in ulcers. This region was termed the plasticity zone, and it was believed that the differences in clinical manifestations were due to the varying genetic composition of the zone. Additional investigators demonstrated that the genetic content of this zone can vary over time in a single human host, further underscoring its plasticity (Israel et al., 2001). Until the sequencing of pVir, it was believe that the plasticity zone genes were *H.*

pylori specific and possibly involved in virulence. Cjp8, Cjp10, Cjp33, Cjp49, Cjp52 share homology with genes present in the plasticity zone of either *H. pylori* strain. The functions of the *H. pylori* protein homologs are all unknown. Mutation of *cjp49*, a homolog of HP1004, resulted in a decrease in invasion equal to 26% of wild-type 81-176 (Bacon et al., 2002). The presence of these orthologous *H. pylori* genes in the pVir plasmid is noteworthy. Since all of the *H. pylori* orthologs present on pVir originate from a region of great inter-strain genetic diversity, this may suggest the horizontal transfer of genes between these two related species or through another species. Plasmid acquisition of DNA sequences has been proposed to be a mechanism for the potentiation of diversity within the plasticity zone of *H. pylori* (Alm et al., 1999; Hofreuter et al., 2002). It is therefore interesting to speculate that the plasticity zone-like region present on pVir might be an area susceptible to rapid genetic change. Such a notion is further reinforced by the identification of the TFSS in *W. succinogenes* and a third recently identified TFSS present in the *H. pylori* plasticity zone (Baar et al., 2003; Kersulyte et al., 2003). The plasticity zone TFSS was found in an archived strain of *H. pylori* J99. This system was absent in the genomic J99 isolate, further underscoring the relative instability of this region. As with the pVir system, both of these system's TFSS genes are flanked by plasticity zone genes/homologs. This suggests that these proteins could potentially facilitate the dissemination of genetic material between these closely related organisms.

Presence of *Campylobacter* Plasmid Specific Genes of Unknown Function

There are 37 genes of unknown function present on pVir. Sixteen of these putative proteins are predicted to be composed of 100 or fewer amino acids and eighteen are

predicted to be between 100-200 amino acids. Mutational analysis of nearly all of the aforementioned genes has not been performed and much work will be necessary to determine their function. Two unknown genes, however, did have an effect on the ability of *C. jejuni* to invade intestinal epithelial cells. Forty seven percent reduction in invasion was demonstrated with a mutantion in *cjp15*, which is predicted to encode a soluble protein of 25.8 kDa (Bacon et al., 2002). Mutation of another gene, *cjp29*, resulted in a decrease in invasion of INT407 cells to levels equal to 15.2% of wild-type. Cjp29 is a glutamine rich protein of predicted size 44.8 kDa with no significant homology to any described prokaryotic proteins. The predicted soluble nature and cytoplasmic localization of both Cjp15 and Cjp29, coupled with their phenotype of reduced invasion, suggest that these proteins may be putative substrates of the TFSS.

Statement of Hypothesis

The heterogeneity exhibited among various *C. jejuni* isolates in *in vitro* invasion levels and virulence in animal models led us to contend that bacterial genetic determinants account for these differences. One of the most substantial differences between *C. jejuni* 81-176 and other *C. jejuni* isolates is the presence of a large non-conjugative plasmid, designated pVir. Sequence and mutational analysis of this plasmid revealed the presence of TFSS homologs, some of which were implicated in intestinal epithelial cell invasion and pathogenicity in the ferret diarrhea model. These observations allowed us to hypothesize that the pVir TFSS was involved in the pathogenesis of *C. jejuni* 81-176 and contributed directly to intestinal epithelial cell invasion by the secretion of protein substrates.

Therefore, a characterization of the pVir TFSS was undertaken that addressed the following aims:

(1) Since *C. jejuni* glycosylates a large proportion of periplasmic and surface proteins, we wished to determine if any of the components of the pVir TFSS was glycosylated. (2) Additionally, we sought to examine if a pVir homolog of a TFSS ATPase, VirB11, was biochemically and structural similar. (3) Lastly, due to lack of information regarding substrates of TFSS, we wanted to attempt to identify candidate substrates of the pVir TFSS.

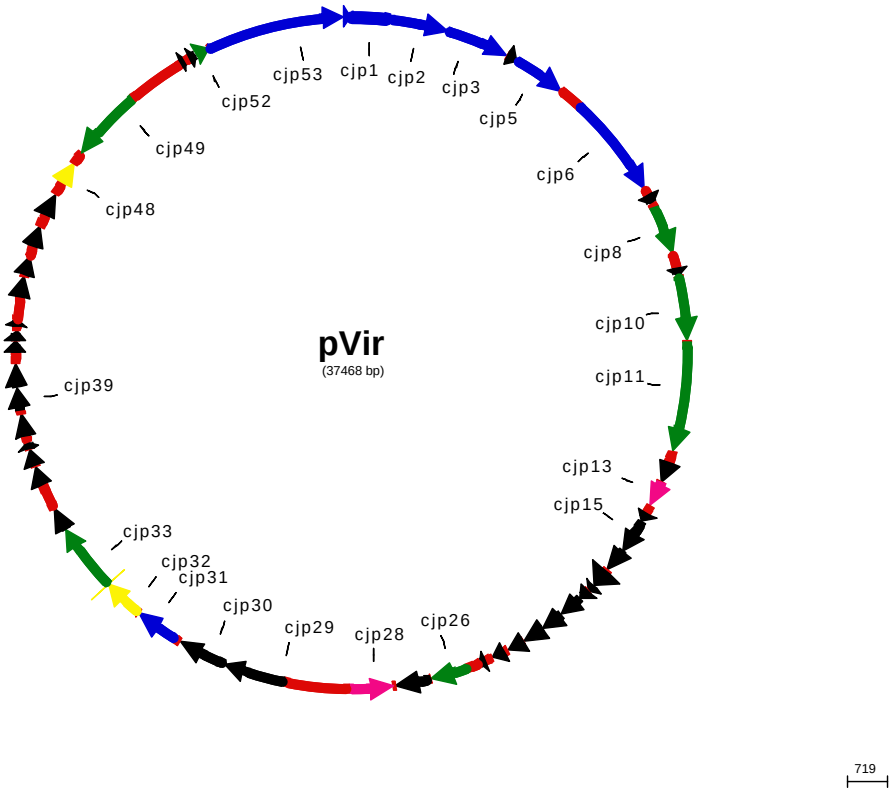

Fig. 1. Circular map of pVir of *C. jejuni* 81-176. Blue arrows represent genes homologous to type IV secretion system genes. Green arrows represent genes with homology to *H. pylori* plasticity zone genes. Magenta arrows indicate genes with similarity to proteins of predicted function. Yellow arrows represent genes with homology to *C. jejuni* NCTC11168 chromosomal genes. Black arrows represent *C. jejuni* plasmid specific genes of unknown function. Red areas indicate non-coding region. Scale indicates length in base pairs.

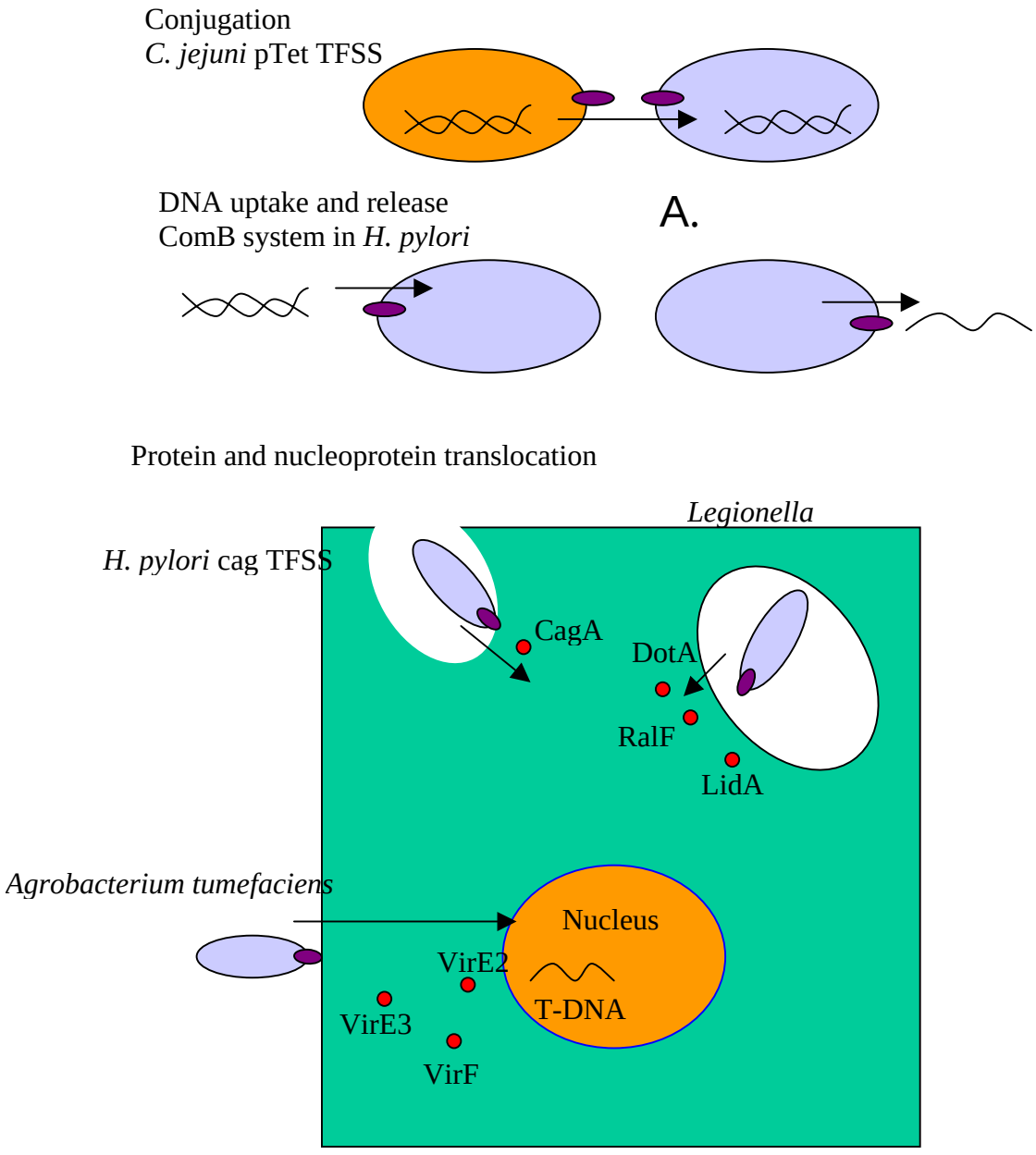

Fig 2. Various functions of TFSS. Figure was adapted from Casacales and Christie, 2003.

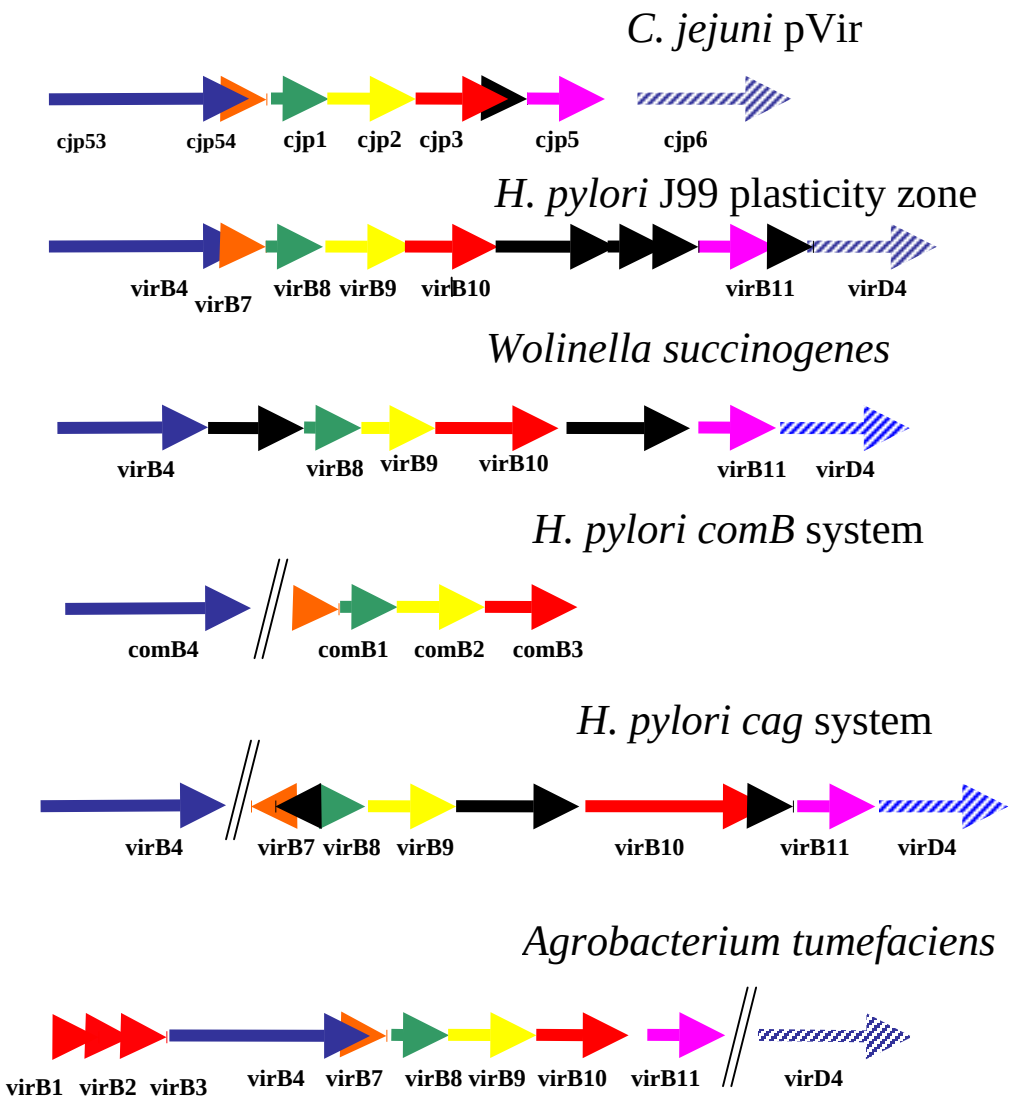

Fig. 3: Genetic composition of TFSS in a number of bacterial species. Identical colors indicate orthologous genes, black arrows indicates genes not related to TFSS genes or genes without orthologs in the selected organisms. Image is not to scale.

pVir TFSS protein (# of amino acids)	*A. tumefaciens*	*W. succinogenes*	*H. pylori* J99 plasticity zone	*H. pylori comB*	*H. pylori cag*	*C. jejuni* 81-176 pTet
Cjp53/VirB4 (822)	24/41 (233)	40/ 63 (778)	30/51 (833)	31/53 (795)	21/39 (289)	21/38 (779)
Cjp1/VirB8 (225)	NS	41/62 (198)	34/57 (223)	35/59 (207)	22/40 (200)	23/40 (176)
Cjp2/VirB9 (356)	NS	38/61 (267)	35/51 (224)	32/52 (287)	NS	24/39 (235)
Cjp3/VirB10 (378)	25/44 (162)	41/58 (329)	35/50 (364)	42/59 (228)	35/53 (115)	29/50 (187)
Cjp5/VirB11 (317)	31/56 (224)	59/69 (287)	40/59 (297)	N/A	34/49 (213)	28/48 (314)
Cjp6/VirD4 (628)	25/44 (309)	36/59 (561)	33/55 (613)	N/A	21/41 (465)	22/41 (488)

Table 1: Comparison of homologies of pVir Type IV secretion proteins with other bacterial species. Results determined by BLASTP analysis. Results presented as percentage of identity/similarity over the length of amino acid homology. Cjp54/VirB7 was omitted from analysis due to non-significant hits by BLASTP, from its small size. CLUSTAL analysis had been previously performed with Cjp54 (Bacon et al., 2002). N/A= not applicable NS=non-significant hit.

CHAPTER 2

Identification of a Glycosylated Structural Component of the pVir TFSS

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Abstract

The recent sequencing of the virulence plasmid of *Campylobacter jejuni* 81-176 revealed the presence of genes homologous to type IV secretion systems (TFSS) that have subsequently been found in *Helicobacter pylori* and *Wolinella succinogenes*. Mutational analyses of some of these genes have implicated their involvement in intestinal epithelial cell invasion and natural competence. In this report, we demonstrate that one of these type IV secretion homologs, Cjp3/VirB10, is a glycoprotein. Treatment with various glycosidases and binding to soybean agglutinin indicated that the structure of the glycan present on VirB10 contains a terminal GalNAc, consistent with previous reports of *N*-linked glycans in *C. jejuni*. Site-directed mutagenesis of five putative *N*-linked glycosylation sites indicated that VirB10 is glycosylated at two sites, N32 and N97. Mutants in the *N*-linked general protein glycosylation (*pgl*) system of *C. jejuni* are significantly reduced in natural transformation, which is likely due, in part, to lack of glycosylation of VirB10. The natural transformation defect in a *virB10* mutant can be complemented *in trans* using a plasmid expressing wild-type VirB10 or an N32A substitution, but not a mutant expressing VirB10 with an N97A substitution. Taken together, these results suggest that glycosylation of VirB10 specifically at N97 is required for the function of the type IV secretion system and for full competence in *C. jejuni* 81-176.

Campylobacter jejuni 81-176 is unique for a prokaryotic organism in that it has a general system of *N*-linked protein glycosylation (*pgl*) affecting a substantial number of periplasmic and surface proteins (Szymanski et al., 1999; Young et al., 2002). The structure of the *N*-linked glycan present on *C. jejuni* NCTC 11168 glycoproteins was recently found to be a heptasaccharide with a mass of 1406 daltons composed of GalNAc-α1,4-GalNAc- α1,4-(Glc-β1,3-) GalNAc- α1,4-GalNAc- α1,4-GalNAc- α1,3-Bacillosamine (Young et al, 2002). The addition of this glycan was dependent on the activity of the PglB protein, which is predicted to function as an oligosaccharide transferase (Szymanski et al., 1999; Wacker et al., 2002; Young et al, 2002) based on its homology to an oligosaccharide transferase subunit (STT3) of *Saccharomyces cerevisiae* (Zufferey et al., 1995). Mutation of certain *pgl* genes, including *pglB*, diminished the ability of 81-176 to invade INT407 cells and colonize the intestinal tracts of mice (Szymanski et al., 2002), reinforcing the importance of protein glycosylation to the pathogenesis of *C. jejuni*. However, the precise functional contribution of *N*-linked glycosylation to the pathogenesis of *C. jejuni* remains unclear.

C. jejuni strain 81-176 possesses two plasmids, one of which, pVir, is non-conjugative and affects both virulence and natural competence (Bacon et al., 2000). Sequence analysis of this plasmid revealed the presence of eight genes with greatest homology to a type IV secretion system (TFSS) subsequently shown to be present in the ruminant commensal *Wolinella succinogenes* (Baar et al., 2003; Bacon et al., 2002). There is also significant homology to two TFSS found in *Helicobacter pylori*.

These are the *com* system which is responsible for natural transformation in *H. pylori* and a more recently described TFSS of unknown function found in the *H. pylori* J99 plasticity zone (Hofreuter et al., 1998, 2001; Kersulyte et al., 2003). In contrast, the pVir TFSS shows much less homology to the well-characterized TFSS found on the *cag* pathogenicity island of *H. pylori* (Censini et al.1996, Tomb et al., 1997). TFSS, which are present in a variety of plant and mammalian pathogens, are involved in the transfer of DNA, protein, or nucleoprotein complexes across bacterial membranes (Cascales and Christie 2003, Christie 2001). The TFSS genes present on pVir have been proposed to encode proteins that form a functional secretion channel that appears to affect both intestinal epithelial cell invasion and natural competence (Bacon et al., 2000, 2002).

Herein, we report that a putative structural component of the pVir TFSS, VirB10 (Cjp3) (Bacon et al., 2002) is glycosylated by the *pgl* system at two asparagine residues and that lack of glycosylation at one site results in a competence defect comparable to a *virB10* insertional mutant. Further, we demonstrate that *pgl* mutants exhibit a major defect in natural competence, suggesting that *N*-linked glycosylation is required for full competence in *C. jejuni* 81-176.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. jejuni* DB179 (81-176 cured of the second plasmid, pTet) has been described (Bacon et al., 2000). All *C. jejuni* mutants used were constructed by insertional inactivation of the target gene by either an *aph3A* cassette (Km^R) (Bacon et al. 2000) or insertion of a *cat* cassette using an

EZ::TN transposon containing a campylobacter *cat* gene (Cm^R) (Bacon et al., 2002). The mutations in the 81-176 *pgl* mutants were Km^R insertions into *pglE* or *pglB* and have been described (Szymanski et al., 1999). The *virB8*::Cm and *virB9*::Cm mutations in pVir were previously described (Bacon et al., 2002). The pVir *virB10*::Km mutation (Bacon et al., 2000) was reconstructed in DB179 to avoid any potential interaction with the conjugative TFSS system encoded by the pTet plasmid also resident in 81-176. All mutants were tested by PCR using primers that bracket the insertion point of the drug resistance gene to confirm a double crossover (Bacon et al., 2000, 2002). *C. jejuni* was grown at 37^{o}C under microaerobic conditions on Mueller-Hinton (MH) agar (Difco). *E. coli* DH5α transformed with the *C. jejuni pgl* genes present on pACYC184 (pBTLPS) was described previously (Wacker et al., 2002). *E. coli* strains were grown on Luria agar. *E. coli* DH5α was used as the host strain for cloning experiments and DH5α containing pRK212.1 was used as the donor in conjugation experiments (Figurski and Helinski 1979) . *E. coli* ER2566 (New England Biolabs) was used as the host strain for protein expression experiments. Antibiotics were added when appropriate to the following concentrations: 100 μg of ampicillin per ml, 20 μg of chloramphenicol per ml, 25 μg of kanamycin per ml, 20 μg of streptomycin per ml, 20 μg of tetracycline per ml, and 10 μg of trimethoprim per ml. Plasmids used are listed in Table 2.

Construction of campylobacter expression vectors. The region upstream of Cj1291, annotated as *accB*, a putative biotin carboxyl carrier protein of acetyl CoA carboxylase (Goon et al., 2003; Parkhill et al., 2000) containing a putative σ^{70} promoter was PCR-amplified using HF2 DNA polymerase (Clontech) with the

following primers: 5' CGGGATCCCGAAAATTCTCCTACAAAATTTAAGAAC-3' and 5' GCTCTAGAGCTTTTAACCTTTTAATATTAGTAATTTTTT-3'. These primers introduced *Bam*HI and *Xba*I sites bracketing the promoter region of Cj1291. The PCR product was digested with *Bam*HI and *Xba*I and cloned into *Bam*HI-*Xba*I digested pRY107, a kanamycin resistant shuttle vector (Yao et al., 1993), to generate pCE107/70. The region upstream of the *flaA* gene containing the σ^{28} promoter was PCR amplified from 81-176 using HF2 DNA polymerase (Clontech, Palo Alto, CA) with the following primers: 5'-GCTCTAGAGCGTAAAATTGAAGATGAAAGAGAG-3' and 5'-CGGGATCCCGTTTTAAATCCTTTTAAATAATTTC-3'. These primers introduced *Xba*I and *Bam*HI sites, respectively. The PCR product was digested with *Xba*I and *Bam*HI (New England Biolabs, Beverly, MA) and cloned into *Xba*I-*Bam*HI-digested pRY111, a chloramphenicol resistant campylobacter shuttle plasmid (Yao et al., 1993) to generate pCE111/28.

Complementation *in trans* of the *virB10* mutation. PCR amplification was used to amplify *virB10* (*cjp*3) from the pVir plasmid using HF2 DNA polymerase (Clontech). The primers to amplify *cjp3*/*virB10* were JCL 075 5'-CGCGGATCCATGAAAAAATCCTTTTTAAGCC-3' and JCL 076 5'-GGCTGCAGTTAATTATCTTGGAAATATTGG -3', which introduced a *Bam*HI and *Pst*I site (5' and 3', respectively) flanking the *virB10* coding sequence. The amplicon was digested with *Bam*HI and *Pst*I and cloned into the *Bam*HI and *Pst*I sites of pCE107/70 or pCE111/28 to create pJL101 and pJL102, respectively. The pJL102 construct and mutant derivatives were mobilized from *E. coli* DH5α containing

pRK212.1 into *C. jejuni* DB179 *virB10*::Km cells. Transconjugants were selected on MH agar containing kanamycin, chloramphenicol and trimethoprim. Plasmid pJL101 was transformed into *E. coli* DH5α with and without pBTLPS (Wacker et al., 2002).

Site-directed mutagenesis of *virB10*. Mutation of five of the six predicted *N*-linked glycosylation sites of VirB10 was carried out using the QuickChange™ site directed mutagenesis kit (Stratagene, La Jolla, CA). Mutations were confirmed by sequencing using a Big Dye™ Terminator sequencing kit (Applied Biosystems, Foster City, CA) on an ABI Prism 3100 genetic analyzer (Applied Biosystems). Oligonucleotides used are listed in Table 3. The construction of the single mutants was carried out using pJL102 as a template and pJL102/N32A was used as the template to construct the double mutant, pJL102/N32, N97A.

Cloning and expression of VirB8, VirB9, and VirB10. The genes encoding VirB8 (Cjp1), VirB9 (Cjp2) and VirB10 (Cjp3) were fused to intein in the expression vector pTYB12 (New England Biolabs) by the following procedure: The DNA fragments were generated by PCR amplification using HF2 DNA polymerase (Clontech). The primers, which introduced *Spe*I and *Eco*RI sites, were *cjp1*-F 5'-GACTAGTGGAATGAGTAATAATACTATTGT- 3', *cjp1*-R 5'-GCGAATTCGTTACTTCGCTCCTTTCGTTTG -3', *cjp2*-F 5'-GACTAGTGGAGACAACATACAAATTCAAGATGTTCC- 3', *cjp2*-R 5'-GCGAATTCGTCATTTCTTAGCCTT -3', *cjp3*-F 5'-GACTAGTGGACAAACAAGCGAAGAAAATGTATC- 3', *cjp3*-R 5'-GGAATTCTTAATTATCTTGGAAATATTGGATCAATA- 3'. The PCR products were digested with *Spe*I and *Eco*RI and cloned into pTYB12 (NEB). The pTYB12-

Cjp1 clone contains an N-terminal intein fusion containing residues 52–225 of Cjp1. The pTYB12-Cjp2 and Cjp3 clones contain residues 22–356 and 28–378, respectively. *E. coli* ER2566 containing these constructs were grown overnight in Luria-Bertani (LB) medium at 37^{o}C. The cells were diluted 1:100 in fresh medium containing ampicillin, and one liter cultures were grown to an OD_{600} of approximately 0.5. Isopropyl-β-D galactoside was added to a final concentration of 0.5 mM, and cultures were grown overnight at 16^{o}C. Bacteria were pelleted by centrifugation at 5,000 Xg for 10 minutes. Cell pellets were stored frozen at -20^{o}C. Affinity chromatography was carried out using the IMPACT-CN ^{TM} protein purification system according to the manufacturer's recommendations (New England Biolabs).

Generation of polyclonal antisera. Protein samples were sent for injection into New Zealand White Rabbits at Harlan Bioproducts (Indianapolis, IN). Following the manufacturer's immunization protocol, polyclonal antisera were obtained and used at the indicated dilution.

Electrophoresis and immunoblotting. *Campylobacte*r and *E. coli* whole cells were resuspended in 1X SDS-PAGE sample buffer to a final protein concentration of 10 μg/μl. Protein samples were aliquoted and resuspended in an equal volume of 2X SDS-PAGE sample buffer. Samples were boiled and loaded onto 10% acrylamide gels. Proteins were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) and detected by staining with Coomassie brilliant blue G250 or, after transfer to nitrocellulose, Western blot analysis using the indicated rabbit antisera. The secondary antibody was goat anti-rabbit antiserum

conjugated to alkaline phosphatase (Caltag, Burlingame,CA) used at a 1:5000 dilution.

SBA affinity columns. Glycine extracts were prepared by resuspending a loopful of campylobacter in 0.2 M glycine-HCl, pH 2.2 and placing it on ice for 10 minutes. Samples were centrifuged at 14,000 rpm and suspended in an equal volume of 2X SDS-PAGE sample buffer. Large-scale glycine extracts were prepared using 100 ml of *C. jejuni* grown in biphasic culture as previously described (Logan and Trust, 1983). Biphasic is a method for growming liguid cultures of *Campylobacter* by placing an MH agar underlay in a tissue cultute flask and putting MH liquid medium over the agar layer. Prepared glycine extracts were incubated with 2 ml of soybean agglutinin (SBA) agarose (Vector Labs, Burlingame, CA) at 4^{o}C. Following the collection of flow through fractions, the column was washed in 20 ml of column buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1 mM $CaCl_2$, and 0.01mM $MnCl_2$) and wash fractions were collected. Proteins were eluted from the column by washing with 3 bed volumes of column buffer containing 0.2 M galactose.

Enzymatic deglycosylation. Neutralized glycine extracts of *C. jejuni* were treated with α-*N*-Acetyl-galactosaminidase or β-*N*-Acetylhexosaminidase (New England Biolabs) according to the manufacturer's recommendations. After the addition of an equal volume of 2X SDS-PAGE loading buffer, samples were boiled and loaded onto a 10% acrylamide gel.

Natural transformation of *C. jejuni*. The biphasic natural transformation procedure was used as previously described (Wang and Taylor, 1990). *C. jejuni* strains were grown overnight on plates and resuspended in MH broth to an OD_{600} of 1.0. Aliquots of 250 μl of

each strain were grown for an additional 2 hours at 37^{o}C in biphasic culture tubes (Guerry et al., 1994 a). DNA (500ng) from a streptomycin-resistant mutant of 81-176 (Guerry et al., 1994 b) was added to cultures, and incubation continued for 4 hours at 37^{o}C. Cultures were serially diluted and plated in duplicate to MH agar containing streptomycin. The results were expressed as the number of transformants per microgram of Str^{r} DNA. Negative controls were treated identically without the addition of DNA.

RESULTS

Discrepancy between predicted and observed mass of VirB10. As shown in Figure 4, lane 1, VirB10 expressed in *C. jejuni* DB179 presents two bands: a major band with an apparent molecular mass of approximately 43 kDa, and a minor band with an apparent molecular mass of 41.5 kDa. The major 43 kDa band is substantially larger than that of the recombinant form of VirB10 expressed in *E. coli* (40.1 kDa) (lane 3) or the predicted mass of the mature VirB10 lacking its signal peptide (40.5 kDa). The significant discrepancy in mass between the recombinant protein expressed in *E. coli* and native forms of VirB10 suggested that VirB10 may be glycosylated in *C. jejuni*.

VirB10 possesses affinity for soybean agglutinin (SBA). Glycine extracts of DB179 contained VirB8, 9, and 10, suggesting a periplasmic or surface localization for these proteins (Figure 5A, B, C, lane 1). Glycine extracts were passed through a SBA-agarose column to bind *N*-linked glycoproteins containing a terminal GalNAc (Linton et al., 2002). Western blot analysis of elution fractions using antiserum against whole cells of 81-176 revealed the presence of multiple proteins, indicating that enrichment of glycoproteins had occurred (data not shown), consistent with

Linton et al. (Linton et al., 2002). When blotted with anti-VirB10 antiserum, a major band corresponding to the native molecular weight (43 kDa) of VirB10 was detected in the elution fractions (Figure 5C, lanes 5-8). The lower 41.5 kDa form of VirB10 also bound to and eluted from the column, but in lower amounts than the 43 kDa form. In contrast, neither VirB8 or VirB9 was detected in the elution fractions using antisera against recombinant forms of these proteins (Figure 5A and B).

VirB10 susceptibility to glycosidases. Glycine extracted proteins of *C. jejuni* DB179 were digested with glycosidases specific for HexNAc. After treatment with α-*N*-acetylgalactosaminidase, which cleaves the internal α1, 3-linked *N*-acetylgalactosamine residue from the rest of the glycan, VirB10 mobility decreased on SDS-PAGE gels (Figure 6, lane 2). Treatment with β-*N*-acetylhexosaminidase, which cleaves terminal β 1, 2, 3, 4, and 6-linked GalNAc and *N*-acetylglucosamine residues, resulted in no discernible difference in mass (Figure 6, lane 3). These data indicate that the glycan present on VirB10 contains an α 1,3-linked GalNAc, consistent with the structure of the campylobacter *N*-linked glycan previously reported (Young et al., 2002).

Restoration of wild-type mobility of VirB10 in *E. coli* expressing the *pgl* system. Recently it was demonstrated that the general protein glycosylation system of *C. jejuni* could be functionally reconstituted in *E. coli* (Wacker et al., 2002). We took advantage of this to provide genetic evidence that VirB10 is a glycoprotein. When VirB10 was expressed *in trans* from pJL101 (a *C. jejuni/E. coli* Km^R expression vector, see Table 2 and Material and Methods) in *E. coli* DH5α in the absence of the *pgl* system, two bands were visible (Figure. 7, lane 3). The major band corresponded

to the mass of recombinant, unglycosylated VirB10 (Figure 7, lane 1) lacking a signal peptide (40.5 kDa). The minor band in these whole cell extracts, of approximately 41.5 kDa, likely represents VirB10 without its leader sequence removed (see below). In DH5α containing both pBTLPS, carrying the intact *pgl* operon (Wacker et al., 2002), and pJL101, bands of similar apparent mass were observed (Figure 7, lane 5), plus an additional band that corresponded to the mass of the glycosylated form of VirB10 expressed in DB179 (Figure 7, lane 6). When a clarified whole cell extract from DH5α (pBTLPS, pJL101) was subjected to column chromatography using the SBA agarose column, the major band detected in the elution fractions (Figure 7, Lane 7), corresponded in mass to that of glycosylated VirB10 expressed in DB179 (lane 6), plus a minor band that had the same apparent mass as the middle band in lane 5 (see below).

VirB10 is not detected in *pglB* or *pglE* mutants. Western blot analysis of glycine extracts of the *pglE*::Km mutant (Szymanski et al., 1999) failed to detect any VirB10 (Figure 8C, lane 3). When the *pglE* mutation was complemented in trans with plasmid pCS101 (Szymanski et al., 1999), VirB10 was detected in the glycine extract (Figure. 8C, lane 4). Some expression of what appeared to be unglycosylated VirB10 was detected in whole cells of *pgl* mutants (data not shown). No detectable differences in expression patterns of VirB8 and VirB9 were observed between wild-type and the *pglE* mutant (Figure 8A and 5B).

Site-directed mutagenesis. VirB10 is predicted to contain 6 potential *N*-linked glycosylation sites (Asn-X-Ser/Thr). A series of site-directed mutagenesis experiments was done on plasmid pJL102, expressing *virB10* under control of the

flaA σ^{28} promoter, to generate asparagine to alanine substitutions in 5 of these 6 possible glycosylation sites. Figure 8 shows a western blot of glycine extracted proteins of the DB179 *virB10*::Km mutant complemented *in trans* with selected mutated derivatives of pJL102. Alanine substitution of N32 of VirB10 (Figure 9, lane 4) resulted in a decrease in mass such that the VirB10 band migrated at a position similar to the minor 41.5 kDa band seen in glycine extracts of DB179 (Figure 9, lane 1). Mutation of N97 of VirB10 resulted in the presence of two equally intense bands (lane 5). The first band corresponded to the minor 41.5 kDa form of VirB10 detected in glycine extracts of DB179 (lane 1) and to that seen in N32A mutant (lane 4). The lower band migrated in parallel to the recombinant unglycosylated form of the protein expressed in *E. coli* (Figure 9, lane 7). When both N32 and N97 were mutated in the same plasmid (lane 6), only one band was detected that was of the same apparent mass as the recombinant, non-glycosylated form of VirB10 (lane 7). Mutation of 3 other putative *N*-linked glycosylation sites of VirB10 (N42, N126, N156) resulted in no discernable difference in mass; representative results for N42A are shown in lane 8. These results suggest the presence of two glycosylation sites within VirB10: N32 and N97.

Effects of glycosylation on natural competence. It was previously reported that mutation of *virB10* resulted in modest reductions in natural competence (Bacon et al., 2000; Wiesner et al., 2003). Since VirB10 is a glycoprotein, *pgl* mutants of 81-176 were tested for natural competence using a chromosomal Str^r marker (Guerry et al., 1994 a). Figure 10 demonstrates that a mutation in either *pglB* or *pglE* resulted in a significant decrease in the number of transformants. *C. jejuni* DB179 produced an

average of 9.3 X 10^4 transformants per microgram Str^r DNA, which translates into efficiencies of 2.35 X 10^{-4} per microgram/cell, which is within previously reported ranges (Wang and Taylor 1990; Wiesner et al., 2003; Wilson et al., 2003). The *pglB* and *pglE* mutants transformed at frequencies of approximately 1.0 X 10^{-8}, an efficiency that is 10,000-fold lower than wild-type. When the *pglE* mutation was complemented *in trans* with pCS101, an increase in the level of competence was observed. The lack of complete complementation likely reflects instability of the plasmid, a phenomenon that has been observed with some genes *in trans* (Guerry, unpublished). The *virB10* mutant was transformed as previously reported (Bacon et al., 2000; Wiesner et al., 2003), at a frequency of 2.3 X 10^{-5} or approximately 10-fold lower than the wild-type strain. The defect in transformation frequency observed in the *virB10* mutant was complemented *in trans* with pJL102 and restored it to wild-type levels. Wild-type levels of competence were exhibited in the mutant containing pJL102/N32A. However, pJL102/ N97A failed to complement the *virB10* mutant, as did pJL102/N32A, N97A encoding a double mutation. These results suggest that glycosylation of VirB10 at N97, but not N32, is essential for wild-type levels of competence in *C. jejuni* 81-176.

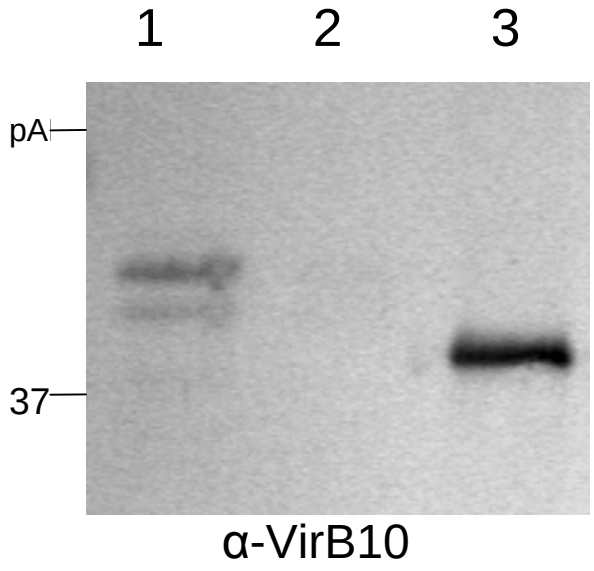


FIG. 4. Discrepancy in mass of VirB10 expressed in *C. jejuni* and *E. coli*. Immunoblot of recombinant VirB10 isolated from *E. coli* and glycine extracts from *C. jejuni* DB179 and isogenic *virB10* mutant. Protein samples were separated on a 10% acrylamide gel. Blots were incubated with an anti-VirB10 antiserum at 1:50,000 dilution. Lane 1, DB179; lane2, DB179 (pVir/*virB10*::Km); lane 3, recombinant VirB10. Numbers indicate protein standards in Kilodaltons (kDa).

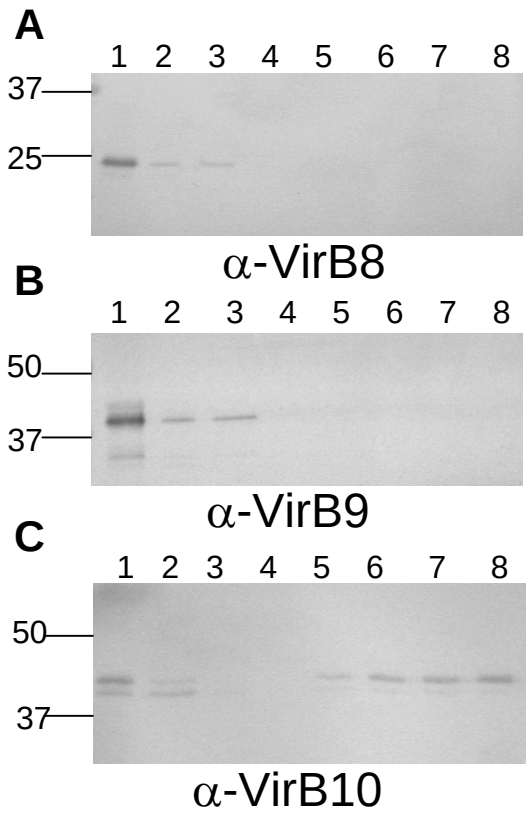


FIG. 5. VirB10 possesses affinity for soybean agglutinin (SBA). Neutralized glycine extracts of DB179 were subjected to column chromatography using a SBA-agarose column. Extract, flow through, wash and elution fractions were subjected to SDS-PAGE, blotted onto membranes and incubated with either (A) anti-VirB8, (B) anti-VirB9, or (C) anti-VirB10 antisera at a 1:50,000 dilution. Lane 1, glycine extract from DB179; lane 2, column flow through fraction; lane 3, wash fraction; lanes 4-8, elution fractions.

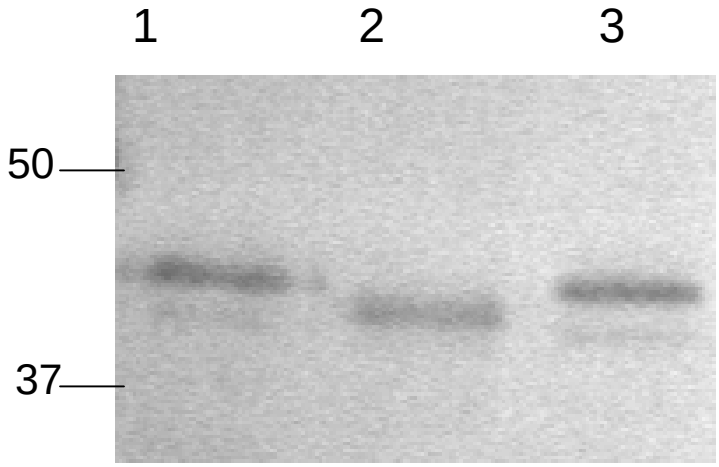


FIG. 6. VirB10 is susceptible to treatment with *N*-acetylgalactosaminidase. Neutralized glycine extracts of *C. jejuni* DB179 were untreated (Lane 1), treated with *N*-acetylgalactosaminidase (lane 2), or β-*N*-acetylhexosaminidase (lane 3). Samples were separated on a 10% acrylamide gel, blotted onto membranes, and incubated with anti-VirB10 antiserum at a 1:50,000 dilution.

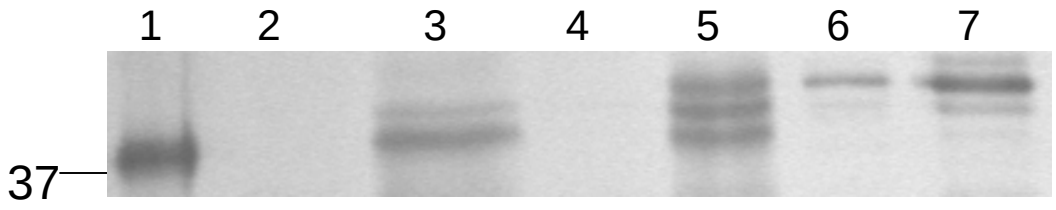


FIG. 7. Analysis of VirB10 expressed in *E. coli* DH5α in the presence or absence of the *C. jejuni pgl* system. Whole cell extracts of *E. coli* were prepared and separated on a 10% acrylamide gel, blotted and immunodetected with VirB10 antisera at 1:100,000 dilution. Lane 1, purified recombinant VirB10; lane 2, DH5α (pCE107/70), the vector alone control; lane 3, DH5α (pJL101), expressing *virB10* in the absence of the *pgl* system; lane 4, *E.coli* DH5α (pBTLPS) containing the *pgl* genes cloned into pACYC184 (Wacker et al., 2002); lane 5, *E. coli* DH5α (pBTLPS, pJL101), expressing *virB10* in the presence of the *pgl* system; lane 6, control of a glycine extract from *C. jejuni* DB179; lane 7, elution fraction from SBA column of lysates from *E. coli* DH5α (pBTLPS, pJL101).

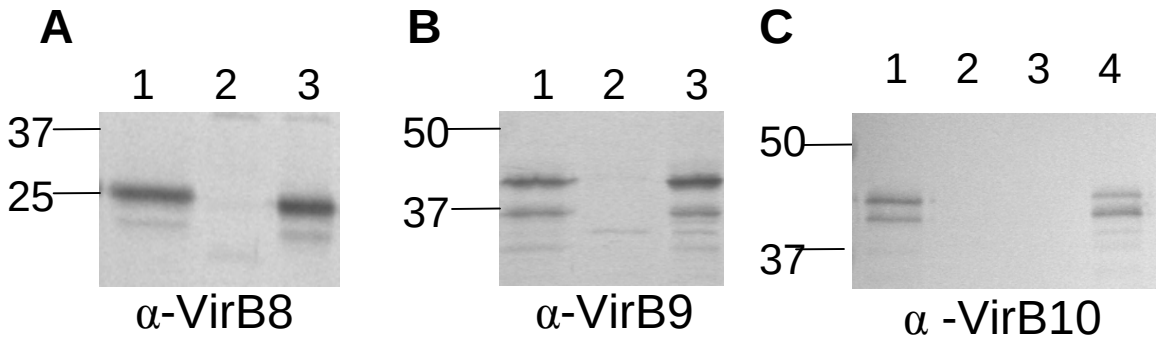


FIG. 8. VirB10 is not detected in the periplasm of mutants defective in the general protein glycosylation pathway of *C. jejuni*. Glycine extracts of *C. jejuni* DB179 and mutants were separated on a 10% acrylamide gel, blotted and membranes were probed with either (A)VirB8, (B)VirB9, or (C) VirB10 antisera at a 1:50,000 dilution. Lane 1: *C. jejuni* DB179; lane 2, 81-176 *virB8*::Cm, 81-176 *virB9*::Cm, DB179 *virB10*::Km (Panel A, B, or C, respectively); lane 3, 81-176 *pglE*::Km; lane 4, 81-176 *pglE*::Km (pCS101) (Szymanski et al., 2002)

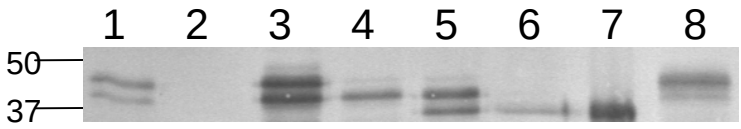


FIG. 9. VirB10 contains two *N*-linked glycosylation sites. Immunoblot of glycine extracted proteins of *C. jejuni* DB179 and mutants. Lane 1: DB179; lane 2, DB179 *virB10*::Km; lane3, DB179 *virB10*::Km (pJL102); lane 4, DB179 *virB10*::Km (pJL102N32A); lane 5, DB179 *virB10*::Km (pJL102N97A); lane 6, DB179 *virB10*::Km (pJL102N32A,N97A); lane 7, recombinant VirB10; lane 8, DB179 *virB10*::Km (pJL102N42A). Anti-VirB10 antiserum was used at a 1:50,000 dilution.

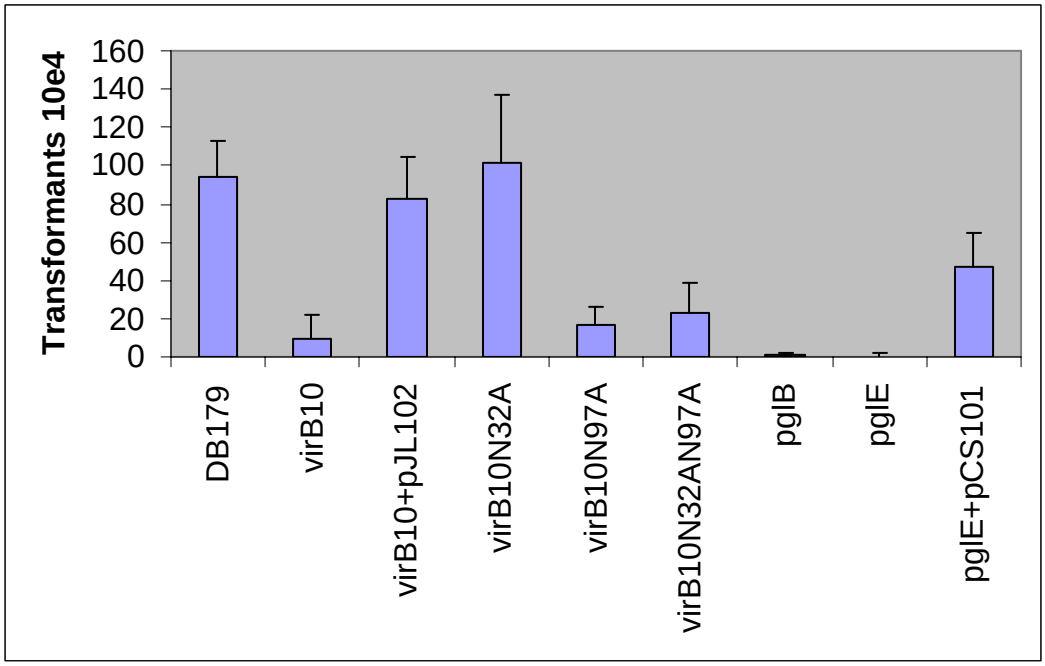


FIG. 10. Contribution of glycosylation to natural transformation. DNA (500ng) from a streptomycin resistant mutant of 81-176 was used to transform *C. jejuni* strains. Results are expressed as the total number of transformants per microgram of DNA and represent the mean and standard deviation of at least three independent experiments. There was no difference in transformation ability of 81-176 and DB179 (data not shown).

Table 2. Plasmids used for the VirB10 analysis.

Plasmid	Description	Reference/source
pBTLPS	Entire *pgl* operon cloned in pACYC184 (Cm^R Tc^S)	Wacker et al., 2002
pCE107/70	*C. jejuni/E. coli* expression vector; derivative of pRY107, a Km^R campylobacter shuttle vector, with σ^{70} promoter from Cj1291	Yao et al., 1993 and this work
pCE111/28	*C. jejuni* expression vector; derivative of pRY111, a Cm^R campylobacter shuttle vector, with σ^{28} promoter from *flaA*	Yao et al., 1993 and this work
pJL101	*virB10* cloned into pCE107/70	This work
pJL102	*virB10* cloned into pCE111/28	This work
pJL102/N32A	N32A substitution in *virB10* coding sequence in pJL102	This work
pJL102/N42A	N42A substitution in *virB10* coding sequence in pJL102	This work
pJL102/N97A	N97A substitution in *virB10* coding sequence in pJL102	This work
pJL102/N32A, N97A	N32A and N97A substitutions in *virB10* coding sequence in pJL102	This work
pCS101	pRY111, Cm^R campylobacter shuttle vector carrying *pglE* under control of *pglE* promoter	Szymanski et al., 1999

TABLE 3: Oligonucleotides used for the site-directed mutagenesis of *C. jejuni virB10*.

Name	Sequence
5’ virB10 N32A	5’gcagaagatatatttgatcaaacaagcgaagaagctgtatctaaaaatatatctaaaaaagacaatcaaagc3’
3’ virB10 N32A	5’gctttgattgtctttttagatatatttttagatacagcttcttcgcttgtttgatcaaatatatcttctgc3’
5’ virB10 N42A	5’cgaagaaaatgtatctaaaaatatatctaaaaaagacgctcaaagccaaaatttgcttaacaaagatttag3’
3’ virB10 N42A	5’ctaaatctttgttaagcaaattttggctttgagcgtctttttagatatattttagatacattttcttcg 3’
5’ virB10 N97A	5’catagtgaagaaaaacctaaaaaagaagaagataatgctattactaagttagcaaaaattgaagaaaaaaag caagaac3’
3’ virB10 N97A	5’gttcttgctttttttcttcaatttttgctaacttagtaatagcattatcttcttctttttaggtttttcttcactatg3’
5’virB10 N126A	5’ cagcaaattgcaaaagaaattcatcaagatgctattagttctcaagaaagaaaaatc 3’
3’virB10 N126A	5’ gatttttctttcttgagaactaatagcatcttgatgaatttcttttgcaatttgctg 3’
5’virB10 N156A	5’ caacacgcaaatttatttttcaagaagcttcaaaatacggcgttgatggtttttc 3’
3’virB10 N156A	5’ gaaaaaccatcaacgccgtattttgaagcttcttgaaaataaatttgcgtgttg 3’

CHAPTER 3

Biochemical Characterization of a Type IV secretion ATPase in *C. jejuni* 81-176

Abstract

Present on the virulence plasmid of *C. jejuni* 81-176 are eight homologs of an *H. pylori* type IV secretion system (TFSS). Mutation of one of these homologs, *cjp5*, results in a significant decrease in intestinal epithelial cell invasion and an attenuation of virulence in the ferret diarrhea model. Cjp5 is homologous to the VirB11 family of ATPases that in other systems provide the energy necessary for substrate secretion through the TFSS. In this work, an initial biochemical and functional analysis of Cjp5 was undertaken. Cjp5 was found to possess ATPase activity and form multimeric structures, consistent with other VirB11 family members. This work represents the first biochemical characterization of a pVir protein and confirms the previous *in silico* predictions regarding Cjp5.

Introduction

Campylobacter jejuni has become well established as a common cause of diarrheal illness in both the developed and developing world, yet details underlying the molecular mechanisms of its pathogenesis remain elusive (Friedman et al., 2000, Oberhelman and Taylor, 2000). Many reports have shown that *C. jeuni* is capable of *in vitro* intestinal epithelial cell invasion, however substantial variations exist in levels of invasion among different *C. jejuni* strains (Everest et al., 1992, Oelschlaeger et al., 1993, Hu and Kopecko, 1999). *C. jejuni* 81-176, a particularly virulent strain that was originally isolated during a diarrheal outbreak associated with the consumption of raw milk (Korlath et al., 1985). This strain was subsequently shown to cause diarrheal illness in two different human challenge studies (Black et al., 1988, D. Tribble, unpublished) and is virulent in the ferret diarrhea model (Yao et al., 1997). It is one of the most intensively studied *C. jejuni* strains and has been shown to invade cultured epithelial cells at level much higher than most *C. jejuni* isolates (Oelschlaeger et al., 1993; Hu and Kopecko, 1999).

C. jejuni 81-176 possesses two plasmids refered to as pVir and pTet. The former plasmid has been directly implicated in virulence (Bacon et al., 2000; 2002). The pVir plasmid contains eight gene homologs of a type IV secretion system present in *Helicobacter pylori* and *Wolinella succinogenes* (Bacon et al., 2002; Baar et al., 2003; Kersulyte et al., 2003). Type IV secretion systems (TFSS) are complex macromolecular structures present in Gram negative bacteria that can secrete DNA, protein, or nucleoprotein complexes into the extracellular space or directly into eukaryotic cells (Cascales and Christie, 2003; Christie 2001). Mutational analysis of

the some of the type IV secretion homologs present on pVir indicated a direct association with intestinal epithelial cell invasion (Bacon et al., 2000, 2002).

In *A. tumefaciens*, VirB11 is an ATPase. VirB11 enzyme activity is required for T-DNA transfer into plant cells (Christie et al., 1989; Stephens et al., 1995). HP0525, the *H. pylori cag* pathogenicity island VirB11 homolog, also has ATPase activity that is essential for the translocation of CagA into gastric epithelial cells and the induction of IL-8 expression (Krause et al., 2000; Backert et al., 2000; Fischer et al., 2001). It is thought that VirB11 proteins reside on the inner face of the cytoplasmic membrane, based upon fractionation experiments and the observation that the *in vitro* ATPase activity of these proteins is increased upon the addition of phospholipids (Krause et al., 2000; Rashkova et al., 1997). These proteins form dynamic hexameric structures that are dependent on the binding of ATP and are believed to provide the mechanistic force necessary to move substrates across the inner membrane (Savvides et al., 2003).

Mutation of Cjp5/VirB11 in *C. jejuni* 81-176 results in significant decreases in intestinal epithelial cell invasion and an attenuation of disease in the ferret diarrhea model (Bacon et al., 2000). Based upon these observations, we propose that Cjp5/VirB11 is functioning in a manner similar to other VirB11 homologs by providing energy for the secretion of TFSS effector proteins. In this work, we characterized Cjp5/VirB11 and demonstrated that this protein is a multimeric ATPase.

Materials and Methods

Bacterial strains, cell lines, and culture conditions. *C. jejuni* DB179 (81-176 cured of pTet) has been described (Bacon et al., 2000). *C. jejuni* was grown at 37^{o}C under microaerobic conditions on Mueller-Hinton (MH) agar (Difco Frankin Lakes, NJ). *E. coli* strains were grown on Luria agar. *E. coli* DH5α was used as the host strain for cloning experiments Ampicllin was added when appropriate to the concentrations of 100 μg per ml.

Cloning and site-directed mutagenesis of *virB11*. PCR was used to amplify *virB11*(*cjp5*) from the pVir plasmid using HF2 DNA polymerase (Clontech Palo Alto, CA). The primers to amplify *cjp5* were JCL 040 5'-CAGGGATCCATGAGTAATAATACTATTGTT-3' and JCL 041 5'-CAGCCTGCAGTTAGATTAATAATTTTGCGTAATC -3', which introduced a *Bam*HI and *Pst*I Site (5' and 3', respectively) flanking the *virB11* coding sequence. The amplicon was digested with *BamHI* and *PstI* and cloned into the *BamHI* and *PstI* sites of pQE80 (Qiagen) to generate pJL106. Plasmid pJL106 was transformed into *E. coli* DH5α for protein expression experiments.

Site directed mutagenesis of *virB11* was performed using the Quickchange™ site directed mutagenesis kit (Stratagene, La Jolla, CA) using pJL106 as the template. Mutations were confirmed by sequencing using a Big Dye™ Terminator sequencing kit (Applied Biosystems, Foster City, CA) on an ABI Prism 3100 genetic analyzer (Applied Biosystems). Oligonulceotides used were JCL064 5' GTCTCAGGTGGAACAGGAAGTGGAGCAACTAGCTTTTTAAATTCTTTAAT

GGG 3’ and JCL065

5’CCCATTAAAGAATTTAAAAAGCTAGTTGCTCCACTTCCTGTTCCACCTGA

G 3’.

Protein purification. DH5α containing pJL106 or pJL106 K156A were grown overnight in Luria-Bertani (LB) medium at 37^{o}C. The cells were diluted 1:50 in fresh medium containing ampicillin and one liter cultures grown to an OD_{600} of approximately 0.5. Isopropyl-β-D galactoside was added to a final concentration of 1.0mM and cultures were grown at 37^{o}C for 4-5 hours. Bacteria were pelleted by centrifugation at 5,000Xg for 10 minutes. Cell pellets were stored frozen at -20^{o}C. VirB11 was subsequently purified using a Ni-NTA agarose column (Qiagen) according to the manufacturer’s protocol. Cell pellets were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole pH 8.0) and after the addtion of lysozyme (1 mg/ml) incubated on ice for 30 minutes. The suspension was subsequently sonicated and centrifuged at 10,000Xg for 30 minutes to clarify the lysate. The lysate was incubated with the Ni-NTA slurry and then washed with 5 volumes of wash buffer (5 0mM NaH_2PO_4, 300 mM NaCl, 50 mM imidazole pH 8.0). Following washing, the fusion protein was eluted from the column using the elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole pH 8.0). Elution fractions containing purified VirB11 were dialyzed overnight against 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.1 mM EDTA, exchanging solution several times, and then concentrated using Amicon centrifugal filter devices (Millipore, Bedford, MA).

Generation of polyclonal antisera. Protein samples were sent for injection into New Zealand White Rabbits at Harlan Bioproducts (Indianapolis, IN). Following the manufacturer's immunization protocol, polyclonal antisera was obtained and used at the indicated dilution.

ATPase assay. The malachite green ATPase assay was performed as previously described (Fan and Macnab, 1996, Lanzetta et al., 1979). Varying concentrations of purified VirB11 were added to 90 μl of 10X reaction buffer (300 mM HEPES, pH 8.0, 300 mM KCl, 300 mM NH_4Cl, 10 mM DTT, 50 mM $Mg[acetate]_2$), 90 μl of 5 mg/ml bovine serum albumin, 36 μl of 100 mM ATP, and 669 μl H_20. After the addition of purified VirB11 (32.5 μg for time course experiments) samples were incubated at 37°C for varying time points or for 30 minutes at increasing VirB11 concentrations. Following incubation, 100 μl of the reaction mixture was added to 800 μl of newly prepared malachite green-ammonium molybdate reagent (three volumes of 0.045% malachite green hydrochloride [Sigma St. Louis, MO], one volume of 4.2% ammonium molybdate tetrahydrate [Sigma] in 4N HCl and $1/50^{th}$ volume of 1% Triton-X-100). After 1 minute at room temperature, 100 μl of 34% citric acid was added to stop color development. After 20 minutes, OD_{660nm} was measured.

Chemical cross-linking, electrophoresis and immunoblotting. The chemical cross-linker Bis-(sulfosuccinimidyl suberate) (BS^3) (Pierce Rockford, IL) was added to a solution of purified VirB11 to a final concentration of 1mM. Following a 30 minute incubation at 37°C, Tris-HCl was added to a final concentration of 50 mM. The samples were incubated for 20 minutes at room temperature and subsequently

prepared for SDS-PAGE. Purified untreated and cross-linked VirB11 was aliquoted and resuspended in an equal volume of 2X SDS-PAGE sample buffer. Samples were boiled and loaded onto 10% acrylamide gels. Proteins were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970) and detected by staining with Coomassie brilliant blue G250 or, after transfer to nitrocellulose, Western blot analysis using VirB11 rabbit antisera at a 1:10,000 dilution. The secondary antibody was goat anti-rabbit antiserum conjugated to alkaline phosphatase (Caltag, Burlingame,CA) used at a 1:5000 dilution. The bound antibodies were detected either by tetrazolium blue and 5-bromo-1-chloro-3-indolyl phosphate in N, N-dimethyformamide (Promega).

Yeast-2-hybrid analysis. Yeast-2-hybrid analysis of VirB11 was performed using the Matchmaker™ Gal4 Two-hybrid system 3 (Clontech). PCR was used to amplify *virB11*(cjp5) from the pVir plasmid using HF2 DNA polymerase (Clontech). The primers used were JCL 051 5'-GGAATTCCATATGATGAGTAATAATACTATTGT-3' and JCL 026 5'-GCGAATTCGTTAGATTAATAATTTTGCGTAATC -3', which introduced a *Nde*I and *Eco*RI Site (5' and 3', respectively) flanking the *virB11* coding sequence. The control and *virB11* plasmids were transformed into yeast strain AH109 (James et. al, 1996) using the Yeastmaker™ yeast transformation system (Clontech) according to the manufacturer's protocol. Strain AH109 was maintained on YPAD media and cultivated at 30°C. Transformed cultures were plated onto Minimal SD agar lacking tryptophan, histidine, and leucine and incubated for 3-4 days at 30°C. Positive clones

were streaked onto SD agars plates lacking tryptophan, histidine, and leucine but containing X-α-galactoside.

Results

A consensus nucleotide binding motif is present in Cjp5/VirB11. Like other VirB11 family members, Cjp5/VirB11 contains a consensus nucleotide-binding motif known as a Walker A box, which is shown in Fig. 11. In order to examine the biochemical and functional properties of Cjp5, site directed mutagenesis was performed. As shown in Fig. 11., a lysine residue within the Walker A box, which has been shown in other TFSS to be critical for function, was substituted with an alanine residue (Krause et al., 2000; Stephens et al., 1995).

Cjp5/VirB11 possesses ATPase activity. The ATPase actvitiy of Cjp5/VirB11 was determined using the malachite green assay. VirB11 was found to contain ATPase activity that increased in both a time and concentration dependent manner (Fig. 12). Purified Cjp5/VirB11 showed an ATPase activity of 9.29+/-0.04 μmol/min of released Pi per microgram of protein. Purified VirB11 K156A, which contains a mutation within the consensus nucleotide-binding site of Cjp5/VirB11, possessed no detectable ATPase activity.

Cjp5/VirB11 forms multimeric structures. Homologs of Cjp5, such as *H. pylori* HP0525, form hexameric structures (Savvides et al., 2003; Krause et al.,2000; Yeo et al., 2000). We therefore sought to provide evidence that Cjp5/VirB11 was capable of self-interaction. Figure 13 shows the qualitative results from a yeast 2-hybrid screen using Cjp5/VirB11 as both bait and prey. Colonies were subcloned on

minimal media lacking leucine, histidine, and tryptophan and containing a chromagenic substrate for a secreted α-galactosidase. The postitive control, detecting an interaction between the SV40 T-Ag and p53 yielded heavy growth and strong positive blue color (Figure 13A), while the negative control, measuring interactions between Cjp5/VirB11 and the eukaryotic extracellular matrix protein laminin, yielded no growth and no blue color (Figure 13C). The self-interaction of Cjp5/VirB11 was readily detected, with clones that had strong growth and a moderate blue color (Figure 13B).

Due to the qualitative evidence gained through the yeast 2-hybrid analysis, we sought to elucidate whether Cjp5/VirB11 was capable of forming multimeric structures. Figure 14 shows the results of an immunoblot of purified VirB11 in the presence or absence of the chemical cross-linker BS^3. In the absence of BS^3, Cjp5/VirB11 migrated to an apparent mass of approximately 37KDa, consistent with its predicted mass. In the presence of chemical cross-linker, however, multiple bands appeared on the gel. The largest band, which migrates slightly above the 200KDa marker, corresponded in mass to that of a hexameric form of VirB11.

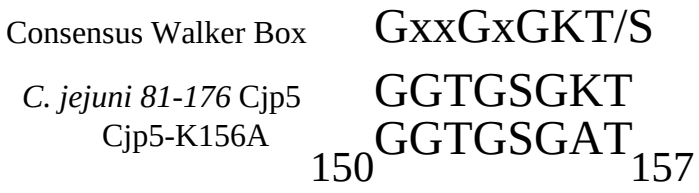
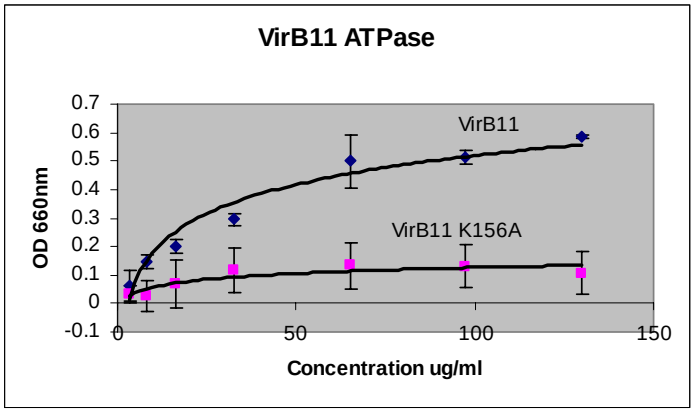


FIG. 11. Mutagenesis scheme for Cjp5. A comparison of the consensus nucleotide-binding motif (Walker A box) with regions of *C. jejuni* Cjp5 and mutant derivative.

A.



B.

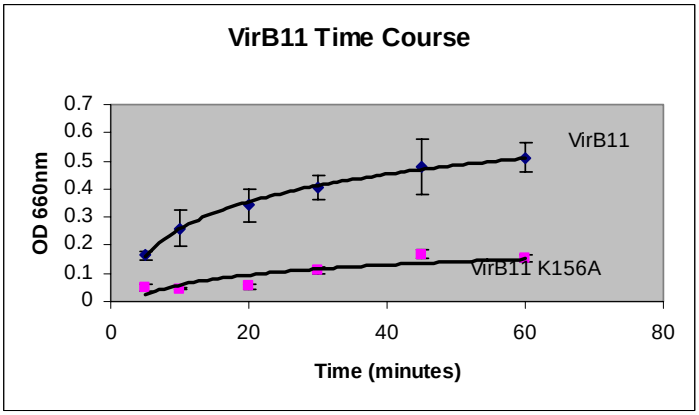
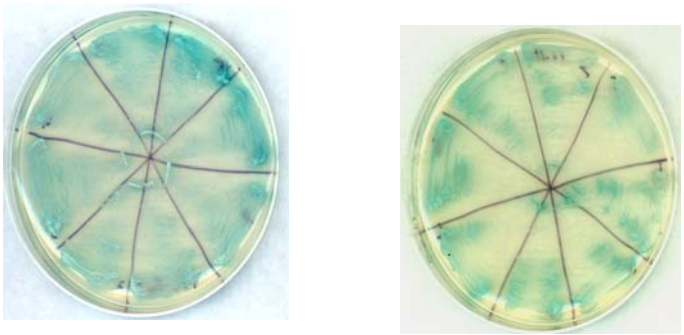


FIG. 12. ATPase activity of Cjp5/VirB11 and Cjp5/VirB11-K156A. Release of inorganic phosphate, measured as a correlate of ATPase activity, was determined using the malachite green spectrophotometric assay (Fan and Macnab 1996; Lanzetta et al., 1979). VirB11 ATPase activity was found to be both concentration (A) and time dependent (B). Values were corrected for background using a buffer only control.

A. B.



C.

FIG. 13.Qualitative yeast 2-hybrid analysis of Cjp5/VirB11. Yeast strain AH109 (Clontech) was transformed with the indicated constructs. Clones positive for growth on Leu- Trp- His- media were patched onto media maintaining nutritional selection and containing a chromagenic substrate for α-galactosidase. (A.) AH109 clones co-transformed with pGDK-SV40-TAg and pGAD-p53. (B.) AH109 clones co-transformed with pGDK-*cjp5* and pGAD-*cjp5*. (C.) AH109 clones co-transformed with pGDK-*cjp5* and pGAD-laminin.

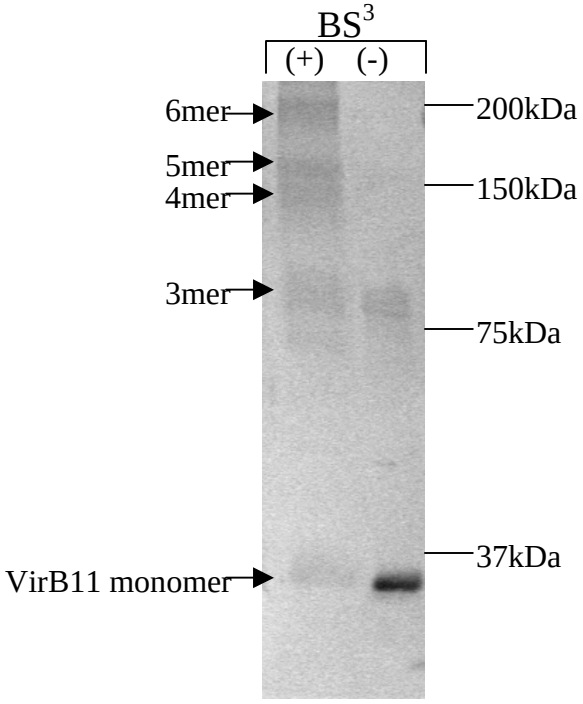


FIG. 14. VirB11 forms multimers. Purified Cjp5/VirB11 was treated with the chemical cross-linked Bis-succinyl suberate (BS^3) and subjected to SDS-PAGE using a 8.25% acrylamide gel. Following transfer to nitrocellulose, filters were incubated with Cjp5/VirB11 polyclonal antisera at a 1:10,000 dilution.

CHAPTER 4

Characterization of pVir-encoded Protein Linked to the Type IV Secretion System

Abstract

Sequence and mutational analysis of the virulence plasmid (pVir) of *C. jejuni* 81-176 revealed the presence of a putative type IV secretion system (TFSS) that was implicated in intestinal epithelial cell invasion. Mutation of another pVir gene, *cjp29*, also reduced 81-176 invasion of intestinal epithelial cells. Here we demonstrate that antibodies against a recombinant form of Cjp29 extensively cross-reacted with numerous eukaryotic proteins, consistent with observed partial homology to eukaryotic proteins by BLASTP analyses. Expression of the Cjp29 protein is co-regulated with the pVir TFSS. Mutations in six TFSS structural genes and one gene of unknown function on pVir were found to have severely decreased levels of Cjp29. Expression of *cjp29* was studied using a combination of arylsulfatase gene fusions and immunoblotting analysis. The 5' non-coding region of *cjp29* contains multiple inverted and direct repeat regions that appear to repress levels of *cjp29* expression. Removal of all DNA except the 289 bp immediately 5' to the start of the gene enhanced expression, but expression was dependent on the presence of a functional TFSS and Cjp29. Homologs of *cjp29* were found in 4/4 clinical isolates of *C. jejuni* that contained a pVir-like plasmid, and sequence analyses of two of these indicated that the predicted proteins were 97% identical to the 81-176 protein. These results suggest that Cjp29 is a protein critically linked to the pVir TFSS.

Introduction

Campylobacter jejuni is one of the major causes of bacteria diarrheal disease in the world. Although the mechanisms of pathogenesis are not well understood at the molecular level, adherence and invasion are thought to be important steps in pathogenesis of *C. jejuni* (Oelschlager et al., 1993, Hu and Kopecko, 1999, Konkel et al., 1999). The levels of invasion of intestinal epithelial cells vary considerably among different strains of *C. jejuni*. *C. jejuni* strain 81-176 was isolated during an outbreak associated with consumption of raw milk (Korlath et al., 1985) and has caused diarrheal disease in two volunteer challenge studies (Black et al., 1988, Tribble and Scott, unpublished). *C. jejuni* 81-176 invades intestinal epithelial cells by a microtubule-dependent process at frequencies that are considerably higher than most other *C. jejuni* strains (Oelschlager et al., 1993, Hu and Kopecko 1999). I*n vitro* invasion appears to correlate with disease in the ferret model of diarrhea (Yao et al., 1993).

C. jejuni 81-176 carries two plasmids (Bacon et al., 2000) both of which have been completely sequenced (Bacon et al., 2000, 2002). One of the plasmids is a tetracycline resistant, conjugative R plasmid, pTet, that does not appear to be involved in invasion (Bacon et al., 2000). Complete DNA sequence analysis of the second plasmid, pVir, which is non-conjugative and 37.4 kb in size, revealed eight homologs of a type IV secretion system (TFSS). TFSS are a diverse group of specialized systems for secretion of DNA, protein, or nucleoprotein complexes (Christie and Vogel 2000, Christie, 2001). Although the pVir encoded TFSS showed homology to many other TFSS systems, it shares its highest similarity with a recently

described TFSS in *Wolinella succinogenes* (Baar et al., 2003). There is also significant homology to two TFSS found in *Helicobacter pylori* (Hofreuter et al., 2001, Kersulyte et al., 2003). These are the *com* system which is responsible for natural transformation in *H. pylori* and a more recently described TFSS of unknown function found in the *H. pylori* J99 plasticity zone (Hofreuter et al., 1998, 2001, Kersulyte et al., 2003). Mutation of some TFSS genes on pVir resulted in a modest reduction in natural transformation of chromosomal genes (Bacon et al., 2000, Larsen et al., 2004, Wiesner et al., 2003) and invasion of intestinal epithelial cells (Bacon et al., 2000, 2002). Additionally, a mutant in a *virB11* homolog (*cjp5*) was attenuated in the ferret diarrheal disease model (Bacon et al., 2000), further suggesting a role for this plasmid in pathogenesis.

A mutation in another pVir gene, *cjp29,* resulted in invasion levels that were about 15% that of the parental wildtype 81-176 (Bacon et al., 2002). Here, we describe our characterization of the Cjp29 protein and show that it is highly cross-reactive immunologically with numerous eukaryotic proteins, and that its expression is dependent on the TFSS. Moreover, homologs of Cjp29 appear to be encoded by pVir-like plasmids in other clinical isolates of *C. jejuni.*

Materials and Methods

Bacterial strains and cell lines. Characteristics of *C. jejuni* 81-176 and mutant derivatives are listed in Table 4. Insertional mutants made with either an *aph3* cassette (*virB10* and *virB11*) or a *cat* cassette into the pVir plasmid of 81-176 have been previously described (Bacon et al., 2000, 2002). *C. jejuni* was grown in

Mueller-Hinton (MH) biphasic medium and on MH agar under microaerobic conditions of 10% carbon dioxide, 5% oxygen and 85% nitrogen at 37°C. *E. coli* strains were cultured on Luria-Bertani (LB) media. *E. coli* DH5α was used as the strain for cloning experiments and for the expression of the MBP-Cjp29 fusion. *E. coli* DH5α (RK212.2) was used to conjugally transfer shuttle plasmids into *C. jejuni*. MH media or LB media were supplemented with antibiotics when appropriate to the following concentrations: 100 μg of ampicillin per ml, 20 μg of chloramphenicol per ml, 25 μg of kanamycin per ml, 20 μg of tetracycline per ml, and 10 μg of trimethoprim per ml.

The human embryonic intestinal cell line (INT407) was obtained from the American Type Culture Collection (ATCC), maintained in liquid nitrogen and cultivated in minimal essential media (MEM) with 10% heat-inactivated fetal calf serum (Gibco BRL), 0.2 mM L-glutamine and nonessential amino acids, as suggested by ATCC (Manassas, VA).

Complementation of *cjp29* mutation *in trans*. A 2.7 kb fragment of pVir that bracketed *cjp29* and included its putative promoter was amplified using primers within the *cjp28* and *cjp30* genes by PCR. The primers, both of which introduced *Sma*I sites, were: *cjp28* F: 5'-TCCCCCGGGCCTGTGGTAAAAAATCTCTAAGTTGCTGG-3" and *cjp30* R: 5'-TCCCCCGGGTCTCTCCATAAAAATCATCTGGCAC-3'. The PCR fragment was digested with *Sma*I and cloned into the *Sma*I site of the kanamycin resistance campylobacter shuttle plasmid pRY107 (Yao et al., 1993) to generate plasmid

pAK101. Plasmid pAK101 was mobilized from *E. coli* DH5α containing RK212.2 (Figurski and Helinski, 1979) into PG1524 (*cjp29*::Cm, see Table 4).

Cloning of *cjp29* homologs from *C. jejuni* strains CG8023 and CG8087. The same primers used to amplify the DNA to complement the *cjp29* mutation in 81-176 (*cjp28* F and *cjp30* R, above) were used to PCR amplify *cjp29* from two isolates of *C. jejuni* obtained from Thailand (Tribble et al., in preparation). The resulting amplicons were cloned into pCRScript (Stratagene) and sequenced using custom primers synthesized on an Applied Biosystems 3400 DNA synthesizer.

Cloning of *virB11*. PCR was used to amplify *virB11* (*cjp5*) from the pVir plasmid using HF2 DNA polymerase (Clontech, Palo Alto, CA). The primers to amplify *cjp5* were JCL 040 5'-CAGGGATCCATGAGTAATAATACTATTGTT-3' and JCL 041 5'-CAGCCTGCAGTTAGATTAATAATTTTGCGTAATC -3', which introduced a *Bam*HI and *Pst*I Site (5' and 3', respectively) flanking the *virB11* coding sequence. The PCR product was digested with *Bam*HI and *Pst*I and cloned into the *Bam*HI and *Pst*I sites of pCE111/28 (Larsen et al., 2004) to create pJL107. The pJL107 construct, which contained *virB11* behind the σ^{28} *flaA* promoter (Larsen et al., 2004), was mobilized from *E. coli* DH5α containing RK212.1 into *C. jejuni* DB179 *virB11*::Km cells to create PG2101.

Overexpression of Cjp29 as a fusion to maltose binding protein (MBP) in *E. coli*. The *cjp29* gene from pVir of *C. jejuni* 81-176 was cloned into the pMalC2 vector of New England Biolabs (Beverly, MA). The gene was PCR amplified with the following primers: 5'-GCTGATATCATGGAAGAAACTAGAGAAGAAACTCC-3' and 5'-

CGCGGATCCTCATAATGACATTCCTTGTCCTTGGC-3'. The resultant 1.1 kb product was digested with *Eco*RV *a*nd *Bam*HI and cloned into pMalC2 that had been digested with *Xmn*I and *Bam*HI. Several clones were subjected to DNA sequence analyses to confirm the correct construction and one was induced with IPTG according to the instructions of the manufacturer. The fusion protein was purified on amylose resin following the supplier's recommended protocols (New England Biolabs). By Coomassie Blue staining the protein sample appeared to be approximately 95% pure.

Overexpression of Cjp29 as histidine tagged protein. The same 1.1 kb fragment (see above) was amplified using primers 5'-ATGGAAGAAACTAGAGAAGAAACTCC-3' and 5'-CTATAATGACATTCCTTGTCCTTGG-3'. The 1.1 kb product was cloned into pCR T7/NT TOPO (Invitrogen, Carlsbad, CA) and several clones were sequenced to confirm the construction. The protein was induced as recommended by the supplier and purified by nickel chromatography, as described above.

Preparation of polyclonal antiserum. Rabbit polyclonal antiserum against the MBP fusion protein of Cjp29 was made in adult New Zealand white rabbits by Harlan Bioproducts Inc. (Indianapolis, IN).

Preparation of affinity purified Cjp29 peptide antigen antiserum. Amino acids 327-344 of Cjp29 were selected following computer analysis by Alpha Diagnostics International for use as the peptide antigen. Peptide synthesis and immunization of adult New Zealand white rabbits was carried out by Alpha Diagnostics International (San Antonio, TX).

Invasion assay. The assay was performed essentially as described previously (Bacon et al., 2002, Hu and Kopecko, 1999). Briefly, about 10^5 IN407 cells per well were added to a 24-well plate, bacteria at multiplicity of infection (MOI) of 20 were added and incubated for two hours at 37°C under 5%CO_2/95% air to allow invasion to occur. Then the infected monolayer was washed three times with Hanks Balanced Salt Solution (HBSS) and incubated another 2h in fresh culture media containing 100 μg/ml gentamicin to kill extracellular bacteria. Subsequently, the infected monolayers were washed three times with HBSS and lysed with 0.1% Triton X-100 in PBS for 15 min. Following serial dilution in phosphate buffered saline (PBS), internalized bacteria were enumerated by plate count on MH agar. All invasion assays were conducted in two separate wells during each assay and were repeated on 3 separate occasions. Data are shown as the mean ± standard error of the mean.

Analysis of protein secretion and translocation. INT407 cells were infected at an MOI=100 in a 100 mm dish with *C. jejuni* 81-176 and mutant derivatives. Following a 4 hour incubation at 37°C, cells were treated as previously reported (Odenbreit et al., 2000). Briefly, cells were washed three times in PBS and then scraped off the dish with 1 ml PBS containing 1 mM $CaCl_2$, 1 mM EDTA, 1 mM orthovanadate, 1 mM PMSF, 1 μM pepstatin A, 1 μM leupeptin. Cells were pelleted are resuspended in 30 μl PBS. Cells were lysed by the addition of PBS containing 0.05% saponin and incubated at room temperature for 10 minutes. Samples were centrifuged for 5 minutes at 6000X g and supernantant separated from the insoluble pellet. The soluble material was filtered through a 0.2 micron filter and then precipitated using the chloroform:methanol procedure as previously described

(Wessel and Flugge, 1984). Both the soluble and insoluble pellets were resuspended in 30 µl SDS-PAGE sample buffer.

Immunoblot analysis. Host cell proteins were subjected to SDS-PAGE as described by Laemmli (Laemmli, 1970). Following transfer to nitrocellulose, membranes were blocked in 2% skim milk in TBST (20 mM Tris-HCl, 150 mM NaCl, pH 7.5, 0.1% Tween 20). The membrane was incubated with polyclonal anti-Cjp29 for 2 hours or overnight at a 1:30,000 dilution. Polyclonal antisera against 81-176 flagellin was used at a 1:500,000 dilution. Alkaline phosphatase (Caltag, Burlingame, CA) anti-rabbit IgG was used as the secondary antibody at a 1:5000 dilution and incubated for one hour. The bound antibodies were detected either by tetrazolium blue and 5-bromo-1-chloro-3-indolyl phosphate in N, N-dimethyformamide (Promega) or by using SuperSignal West Pico Chemiluminescence substrate (Pierce).

Construction of *astA* reporter gene fusions. The intergenic region between *cjp28* and *cjp29* was PCR amplified with primers that introduced *Sac*II and *Xba*I sites, respectively. The primers used were: JCL 116 5'-TATCCGCGGGTGATATACAAGATTGTTATTTAT-3' and JCL 117 5'-GCTCTAGATGTTATGATCCTTTAAAATAATTA-3'. The resulting PCR fragment was digested and cloned into the *Sac*II and *Xba*I sites of pRY107 (Yao et al., 1993) to create pJL103. The *C. jejuni* 81-176 arylsulphatase gene (*astA*) was PCR amplified with primers that introduced *Xba*I and *Kpn*I sites, respectively. The primers used were: AstF 5'-GCTCTAGAGCATGAGACTTAGCAAAACTCTTTGTATG-3' and AstR 3'-

GGGGTACCCCTTATTTTTTAGGATTGAATGCTTGATC**.** Following digestion, the PCR fragment was cloned into the *Xba*I and *Kpn*I sites of pJL103 to create pJL104. The 858 base pair deletion of the *cjp29* intergenic region was generated by digesting pJL104 with *Sac*II and *Cla*I, blunting the resulting fragment with Klenow and self-ligating to create pJL105.

Arylsulphatase assay. *C. jejuni* whole cells were assayed for arylsulphatase activity using previously reported methods (Henderson and Milazzo, 1979, Hendrixson and DiRita, 2003, Yao and Guerry, 1996). *C. jejuni* strains were grown overnight, taken from plates and resuspended in PBS to an OD_{600nm} of 0.6-0.8. Samples were split into two 1 ml aliquots and washed with one ml of either arylsulphatase buffer 1 (0.1 M Tris, pH 7.2) or arylsulphatase buffer 2 (2 mM tyramine, 0.1 M Tris, pH 7.2). Samples were resuspended in one ml of the respective buffer. Two hundred microliters of each sample were added to 200 µl of arylsulphatase buffer 3 (20 mM nitrophenylsulphate, 0.1 M Tris, pH 7.2) and samples were incubated at 37°C for 1 hour. Reactions were stopped by the addition of 800µl 0.2 N NaOH and activity determined by spectrophotometric measurement at 410 nm. Samples resuspended in arylsulphatase buffer 1 served as blanks for samples resuspended in buffer 2. A standard curve of known nitrophenol concentrations was used to determine the amount of nitrophenol released in bacterial assays. One arylsulphatase unit is defined as the amount of enzyme catalyzing the release of 1 µmol of nitrophenol per hour per OD_{600nm} of 1.0 (Adachi et al., 1974).

Construction of a *cjp29* mutant in *C. jejuni* CG8023. Plasmid DNA was harvested from PG1524 using previously published protocols (Bacon et al., 2000,

2002). The resultant DNA was used to electroporate *C. jejuni* CG8023 as previously described and transformants were selected on MH media containing chloramphenicol (Miller et al., 1988, Guerry et al., 1994b, Guerry et al., 2000). The resultant strain was designated PG2177. Transformants were analyzed using primers flanking the insertion point to confirm that a double cross over had occurred.

Accession numbers. The sequences of *cjp29* from CG8087 and CG8023 were deposited in Genbank as AY730722 and AY730723, respectively.

Human use. The human sera used in this study were obtained under NMRC protocol. Protocol received ethical review and approval through the U.S. Army Medical Research Institute of Infectious Diseases (FY97-2) and the U.S. Army Human Subjects Research Review Board (Log No. A-7707).

Results

Homology of Cjp29 to eukaryotic proteins. Cjp29 encodes a predicted protein of 373 amino acids that is rich in glutamic acid (20.6%) and has a pI of 4.67 (Bacon et al., 2002). Cjp29 lacks a leader sequence and is predicted by PSORT and other algorithms to be cytoplasmic, a location that is consistent with Western blot analysis of soluble and membrane fractions of *C. jejuni* 81-176 with a rabbit polyclonal antiserum generated against recombinant Cjp29 (data not shown). Although it shows no significant homology to known proteins by BLASTP analysis (Bacon et al., 2002), BLAST analysis at the NCBI human blast site, which contains human sequences not yet present in the general Genbank database, (www.ncbi.nlm.nih.gov/genome/seq/page.cgi?F=HsBlast.html&&ORG=Hs) revealed

significant homology to a central region of the human CENP-E protein, a kinesin family member that is involved in movement of chromosomes during mitosis. This homology (24% identity and 45% similarity) was limited to amino acids 1003-1381 of the 2663 amino acid CENP-E protein, however. The region of homology includes a conserved ATPase domain of the Smc family of proteins, which is involved in eukaryotic chromosome segregation (Weaver et al., 2003). Relatedness of Cjp29 with eukaryotic cell proteins is also evidenced by the extensive amount of cross-reaction between antibodies directed against a recombinant form of Cjp29 and INT407 cells by Western blot (see below).

Complementation of the Cjp29 mutant *in trans*. In a preliminary screen of pVir mutants, it was shown that a Cjp29::*cat* mutant invaded INT407 cells at about 15% of wildtype levels (Bacon et al., 2002). In order to confirm that this invasion defect was due to the mutation in Cjp29 and not to a polar effect on the downstream gene or phase variation at an unknown site (Parkhill et al., 2000), the mutant was complemented *in trans* with a shuttle plasmid called pAK101, containing the *cjp29* gene and all of the intergenic region between *cjp28* and *cjp29* gene. The strains were characterized by immunoblot analysis using a polyclonal rabbit antiserum raised against a fusion of Cjp29 to maltose binding protein. The antiserum detected 2 proteins in 81-176 of apparent masses approximately 48 and 51 kDa, as shown in Fig. 15A. The predicted mass of Cjp29 is 44.7 kDa (Bacon et al., 2002), but the band running at approximate M_r of 51 kDa was absent in the *cjp29* mutant (PG1524) and was restored in the complement, PG1874. The band at approximately M_r 48 kDa was unaffected by mutation of *cjp29* and likely represents an unidentified

cross-reactive protein. *C. jejuni* is known to glycosylate numerous proteins and this glycosylation can result in aberrant gel migration (Thibault et al., 2001, Linton et al., 2002, Wacker et al., 2002, Larsen et al., 2004). However, there was no difference in the apparent mass of Cjp29 in 81-176 mutants defective in either *N*- or *O*-linked glycosylation (data not shown; Szymanski et al., 1999, Goon et al., 2003). Moreover, a recombinant form of Cjp29 with a 6-His tag also ran at an apparent M_r of 51 kDa (data not shown), suggesting that the aberrant mobility is likely a reflection of the high levels of glutamic acid.

Fig. 15B shows the relative invasion efficiency of *C. jejuni* 81-176, *cjp29*::*cat* (PG1524), and the mutant complemented *in trans* with pAK101 (PG1874). In these experiments, the *cjp29* mutant invaded at about 17.1% the level of wild-type and the complemented mutant invaded at 94.0%, suggesting that the reduced levels of invasion were due to mutation of *cjp29*.

Expression of Cjp29 in 81-176 is linked with the putative TFSS of pVir. Immunoblots of bacterial whole cell proteins were probed with polyclonal anti-Cjp29 antibodies (Fig. 16) to examine Cjp29 expression in a collection of pVir mutants (Bacon et al., 2002). Expression of Cjp29 was significantly attenuated in all mutants of the putative type IV secretion genes encoded on the plasmid (*virB8-11, virB4, virD4*), as well as *cjp51*, a pVir gene of unknown function that maps just upstream of the TFSS genes (Bacon et al., 2002). A representative blot is shown in Fig. 16A. To control for sample loading, the same samples were immunodetected using an antibody generated against 81-176 flagellin (Fig. 16A). Strains with mutations in other pVir genes, *cjp8, cjp11, cjp12, cjp13, cjp15, cjp16, cjp23, cjp24, cjp26, cjp30,*

cjp32, *cjp49*, which are not homologous to any TFSS component, all displayed wild-type levels of Cjp29 expression (Fig. 15 and data not shown). Expression of VirB8, VirB9 and VirB10 was not affected, however, in a *cjp29* mutant as determined by Western blot (data not shown) using antisera directed against recombinant forms of these proteins (Larsen et al., 2004). Fig 16B shows that expression of Cjp29 in a *virB11*::Km background can be restored when *virB11* is provided *in trans*. These data suggested that an intact TFSS was required for expression of the Cjp29 protein, which led us to further study this unusual dependence on a TFSS.

Analysis of intergenic region preceding *cjp29*. The intergenic region between *cjp28* and *cjp29* contains multiple direct and inverted repeats (Bacon et al., 2002), which are represented schematically in Fig 17. These repeats are included in the DNA cloned in pAK101 for the complementation study (Fig. 15). This intergenic region was fused to *astA* on a shuttle plasmid (pJL104) to examine the regulation of *cjp29* with the TFSS (Yao and Guerry, 1996, Hendrixson and DiRita, 2003) and the results are shown in Figure 17. Fusions with *astA* were chosen due to previously reported problems with using l*acZ* fusions in *C. jejuni* (Hendrixson and DiRita, 2003) Surprisingly, fusion of the entire 1,147 bp *cjp29* intergenic region to *astA* in pJL104 resulted in no detectable levels of AstA activity in either DRH461, an *astA* deletion mutant of 81-176 or NCTC 11168, which lacks the *astA* gene (Hendrixson and DiRita, 2003, Parkhill et al., 2000). Deletion of 858 base pairs of the noncoding region, which eliminated the multiple repeat region and left only 289 bp 5' to *astA* (plasmid pJL105), resulted in *astA* levels of 39.5 +/- 0.54 enzyme units in DRH461. Interestingly, pJL105 displayed no detectable activity in NCTC 11168.

However, the *astA* activity of this fusion was restored in PG1777, a NCTC 11168 derivative harboring a tagged version of the pVir plasmid encoding a functional TFSS. Constructions containing either pJL104 or pJL105 did not express any detectable AstA activity in the DRH461 background with a mutation in the TFSS (*virB9*::Cm), suggesting that the regulation with the pVir TFSS is imparted only within the last 289 bp of the *cjp29* intergenic region. Additionally, mutants in *cjp29* did not display AstA activity from either pJL104 or pJL105, suggesting that Cjp29 may be involved in the regulation of its own expression. Moreover, the presence of the repeat region in pJL104 appeared to repress gene expression to levels not detectable by *astA* fusion.

Attempts to detect Cjp29 translocation into INT407 cells. Since Cjp29 was shown to be co-regulated with the pVir TFSS, we hypothesized that Cjp29 was an effector that was translocated into INT407 cells. *C. jejuni* 81-176 and the *cjp29* mutant PG1524 were used to infect INT407 cells for 2 hours. Using a differential saponin lysis procedure followed by methanol:chloroform precipitation (see Materials and Methods), immunoblots using polyclonal Cjp29 antisera were performed. As shown in Fig 18, there was extensive cross-reactivity using the Cjp29 antisera against eukaryotic cell proteins, whereas the pre-immune rabbit serum did not show cross-reaction (data not shown). In Fig. 18, Lane 2, there is a faint band corresponding to the predicted size of Cjp29 in the saponin insoluble fraction of cells infected with *C. jejuni* 81-176. This band is absent from lanes from uninfected cells or cells infected with PG1524 (Fig 18, Lanes 1 and 3). In the saponin soluble fractions, the pattern of bands was identical in all samples, (Fig 18, Lane 4-6) although there were numerous

cross-reactive bands in the region where Cjp29 would be predicted to migrate. This suggested that Cjp29 was either not translocated inside INT407 cells or the levels of Cjp29 secreted are below the limits of detection due to the high levels of background. To circumvent the problems with cross-reactivity, antiserum against a synthetic Cjp29 peptide antigen was generated. This antibody, which was subsequently affinity purified, had lower cross-reactivity to eukaryotic proteins, but had reduced reactivity to Cjp29 and, thus, was not sensitive enough to perform translocation experiments (data not shown). Cjp29 was also not detected in concentrated bacterial culture supernatants or cell culture supernatants following infection (data not shown).

Convalescent human sera detect components of the pVir TFSS. To investigate whether Cjp29 is immunogenic during human infection, we performed immunoblots using convalescent human sera obtained from a volunteer feeding of 81-176 (Tribble and Scott, unpublished). Human volunteers were pre-screened by ELISA against glycine extracts of *C. jejuni* 81-176 to determine if previous exposure had taken place. Individuals with ELISA results of OD_{650nm} greater than 0.0005 were excluded from study participation. A representative blot using post-infection serum is shown in Fig. 19. Positive reactions to glycine extracts were detected in all five serum samples tested, suggesting all individuals immunologically responded to *C. jejuni* 81-176 infection (Fig 19). However, sera from all 5 individuals failed to react with recombinant Cjp29. Interestingly, four of the five serum samples had a positive reaction to a recombinant form of a structural component of the pVir TFSS, VirB10 (Fig. 19). No significant responses were detected to recombinant forms of two other pVir TFSS proteins, VirB8 and VirB11 (Fig 19).

Presence and heterogeneity of Cjp29 in clinical isolates of *C. jejuni*. It has previously been reported that other clinical isolates of *C. jejuni* contained pVir-like plasmids (Bacon et al., 2000, Leonard et al., 2003, Schmidt-Ott et al., 2005). Four clinical isolates from Thailand that were probe positive for *virB11* and *cjp29* (data not shown) were immunoblotted with anti-Cjp29 antiserum. A representative blot for one isolate, CG8023, is shown in Fig 20. The Cjp29 antibodies recognized a protein that migrated at an apparent mass approximately 10 kDa larger than Cjp29 expressed in 81-176 (Fig 20. lane 3). To confirm that this immunoreactive band was Cjp29, a *cjp29*::Cm mutant was reconstructed in the CG8023 background. As shown in Fig 20 lane 4, the 60 kDa immunoreactive band was absent, suggesting it is Cjp29. Identical patterns of electrophoretic mobility were seen in the 3 other independent clinical isolates (data not shown). Sequence analysis of *cjp29* homologs from two strains, CG8023 and CG8087, revealed that the two Thai strains encoded predicted Cjp29 proteins that were identical to each other and 97% identical to the 81-176 homolog. Only eleven amino acid changes were noted when the sequences were aligned by CLUSTAL analysis (Fig 20).

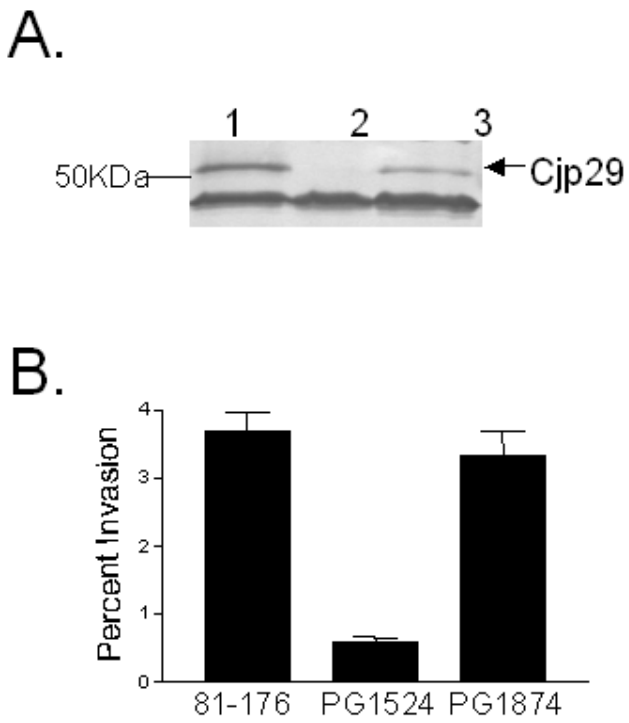
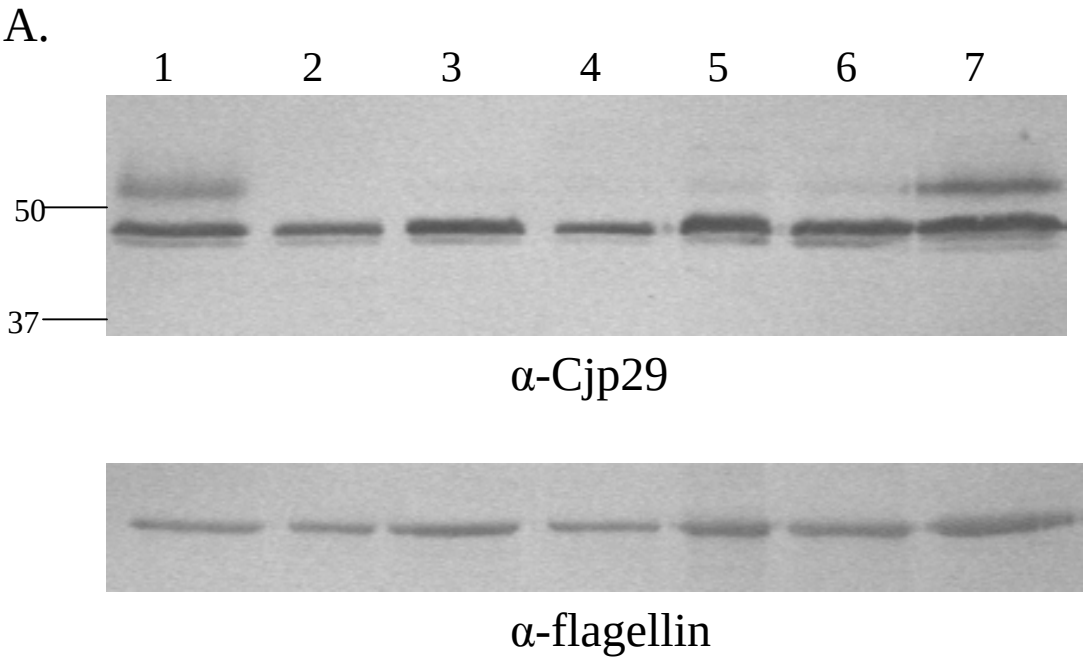


FIG. 15. (A) Complementation of *cjp29* mutant. Whole cell lysates of *C. jejuni* 81-176 and derivatives were immunoblotted with Cjp29 antisera as described in Materials and Methods. Lane 1). *C. jejuni* 81-176. Lane 2). PG1524. Lane 3). PG1874. (B) Relative invasion of INT407 cells. A monolayer of approximately $1X10^5$ INT407 cells was infected at an MOI of 20 with *C. jejuni* 81-176 and derivative strains. Invasion was determined by plate count enumeration following gentamicin treatment. Results are expressed as the decrease in invasion relative to wild-type 81-176 and are the results of three independent experiments. Error bars represent +/- one standard deviation.



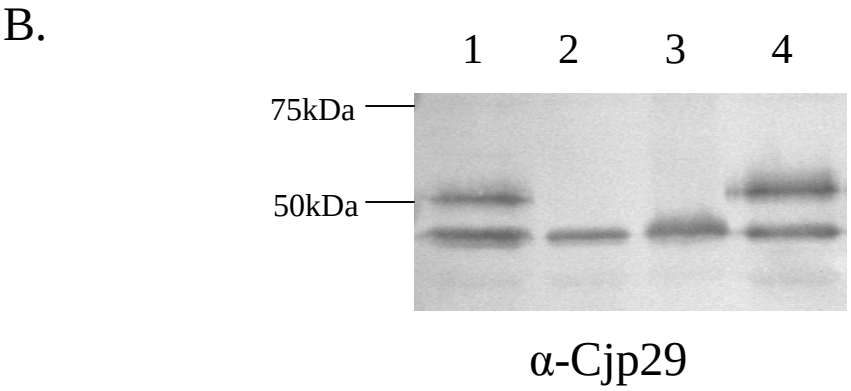
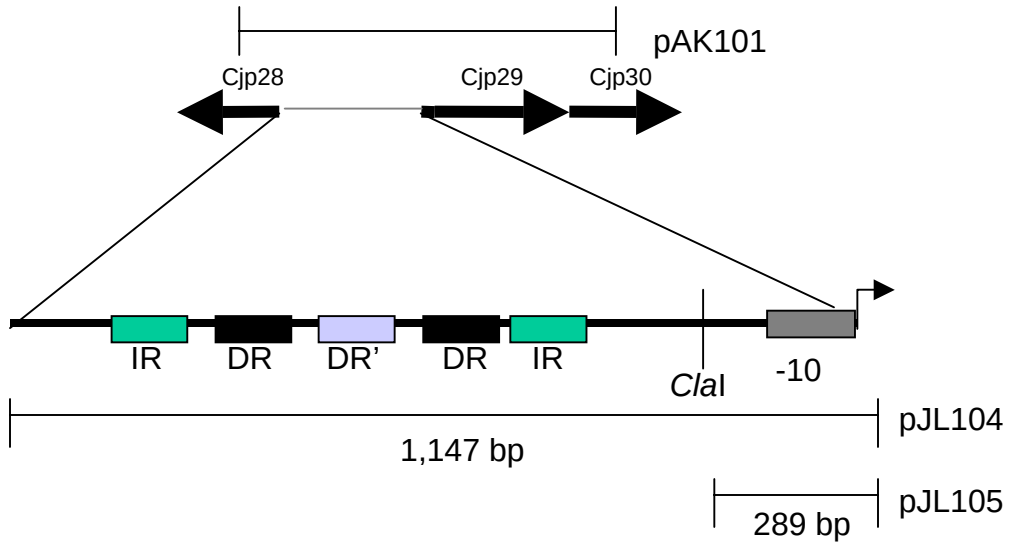


FIG. 16. Cjp29 exhibits TFSS-dependent expression. (A)Whole cell lystates of *C. jejuni* 81-176 (Lane 1) and *cjp29*, *virB8*, *virB9*, *virB10*, *virB11*, and *cjp49* mutant strains (Lanes 2-7, respectively) were immunoblotted and probed with either polyclonal Cjp29 or flagellin antisera at the indicated dilution (see Material and Methods). (B) Complementation of negative *cjp29* regulation. Whole cell lystates of *C. jejuni* 81-176 (Lane 1), *cjp29* (Lane2), *virB11* (Lane 3) mutant strains, and *virB11*::Km +pJL107, carrying VirB11 *in trans* (Lane 4).

A.



B.

Strain Background	pVir	Presence of a functional TFSS	Presence of *cjp29*	AstA units*	
				pJL104	pJL105
DRH461	(+)	(+)	(+)	(-)	39.5 +/-0.54
NCTC 11168	(-)	(-)	(-)	(-)	(-)
NCTC11168+(pVir/ *cjp11*::cat)	(+)	(+)	(+)	(-)	64.6 +/-1.93
DRH461 pVir/*virB9*::cat	(+)	(-)	(+)	(-)	(-)
NCTC11168+(pVir/ *cjp29*::cat)	(+)	(+)	(-)	(-)	(-)

FIG. 17. (A) Diagram of intergenic region between *cjp28* and *cjp29*. IR= inverted repeats (30 bp) DR=perfect 52 bp direct repeats. DR'= imperfect direct repeat (36 of 52 DR bp). The DNA sequence of the repeats is given in Bacon et al., 2002. Arrowed regions indicate sections of the *cjp29* 5' noncoding region used to construct pJL104, pAK101,or pJL105 as described in Materials and Methods. (B) Results from *astA* fusions in various *C. jejuni* backgrounds. (-) = No detectable activity. *One unit equals the amount of AstA needed to generate 1µmol of nitrophenol per hour per OD_{600nm} of 1.0. *C. jejuni* 81-176 had an activity of 77.6 +/- 0.45 AstA units. *C. jeuni* DRH461 and NCTC11168 had no detectable AstA activity as previously reported (Parkhill et al., 2000, Hendrixson and DiRita 2003).

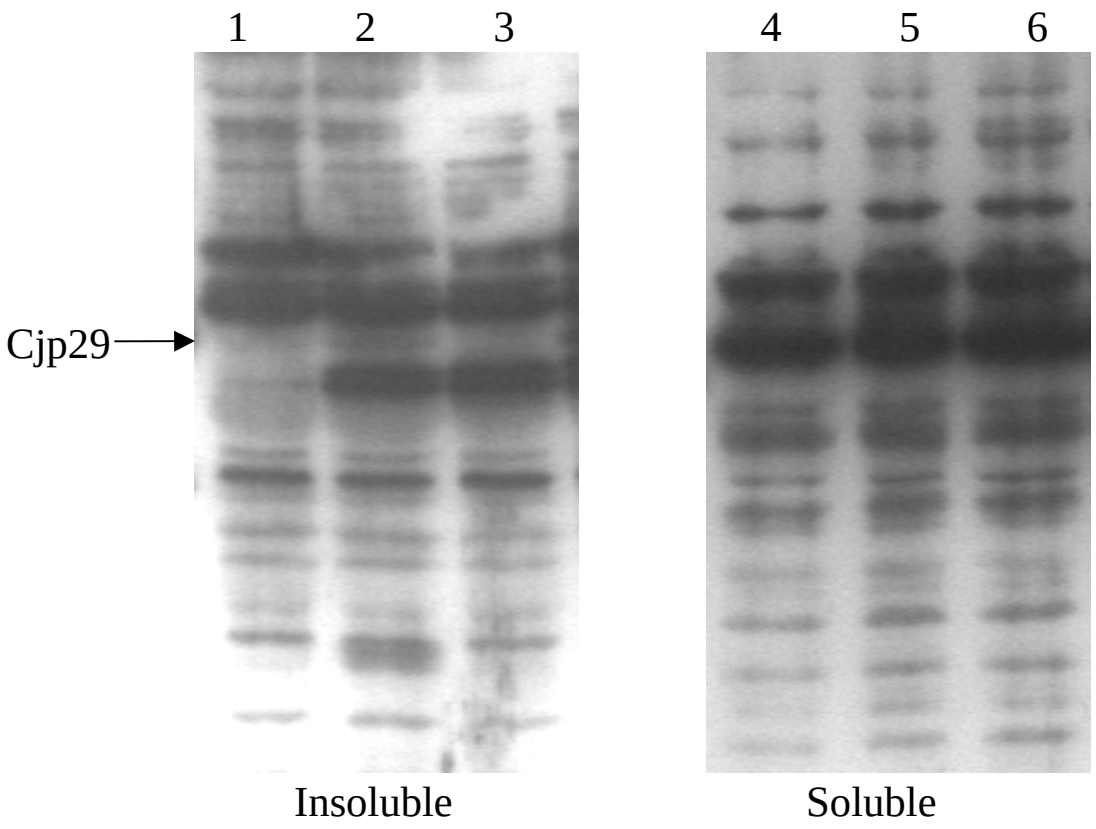


FIG. 18. Analysis of Cjp29 translocation into INT407 cells. Western blots of INT407 cells lystates using ploYclonal Cjp29 antisera following infection with *C. jejuni* 81-176 or PG1524. 100mm dishes of INT407 cells were infected with *C. jejuni* and mutant derivatives and treated according to the protocol outlined in Materials and Methods. Lanes 1-3: Saponin insoluble fraction of uninfected cells. (Lane 1), cells infected with *C. jejuni* 81-176 (Lane 2), or PG1524 (Lane 3). Lane 4-6: Saponin soluble fraction of uninfected cells (Lane 4), cells infected with *C. jejuni* 81-176 (Lane 5), or PG1524 (Lane 6). Preimmune rabbit sera did not display reactivity to eukaryotic cell lysates (data not shown).

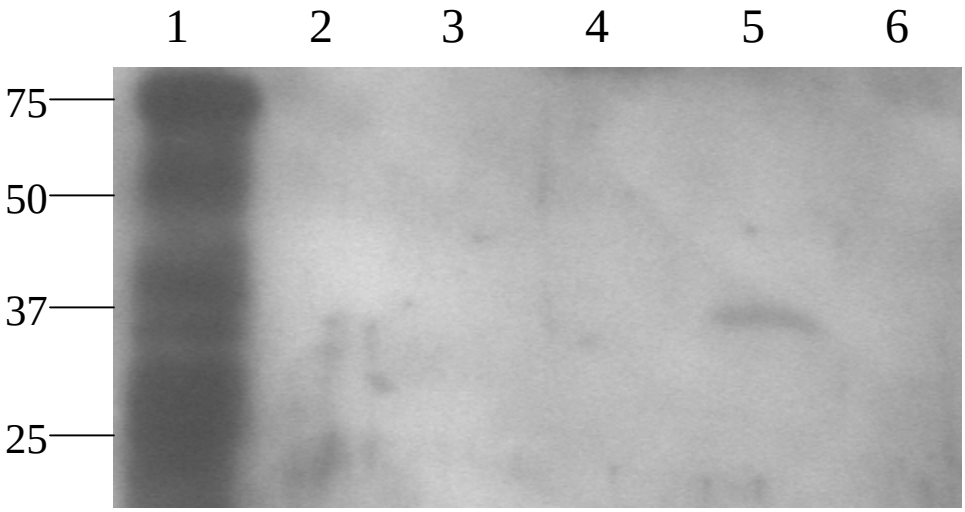
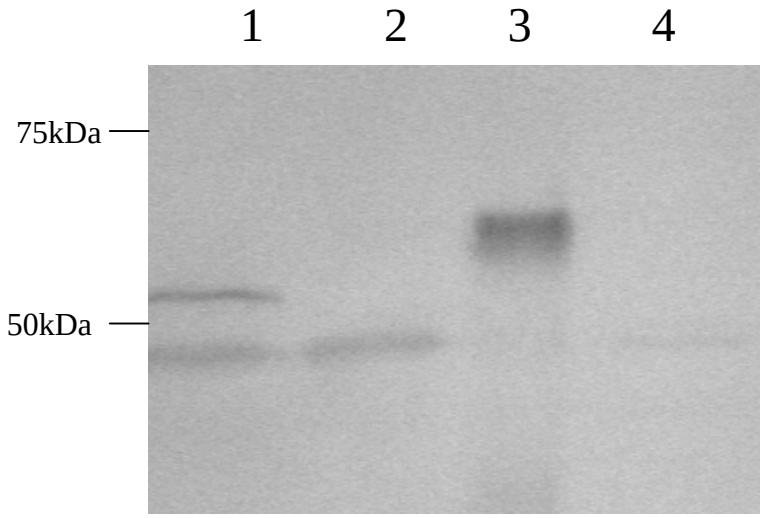


FIG. 19. Human convalescent sera recognizes VirB10 of the pVir TFSS. Recombinant proteins were immunblotted and probed with convalescent human sera at a 1:10,000 dilution. Lane 1: Glycine extract from *C. jejuni* 81-176. Lane2: Bovine serum albumin. Lane 3: Cjp29. Lane 4: VirB8. Lane 5: non-glycosylated VirB10. Lane 6: VirB11. Sera of human volunteers were pre-screened by ELISA using *C. jejuni* 81-176 glycine extracts (which includes the pVir TFSS). Exclusion criteria was defined as any individual that had an OD_{650nm} of greater than 0.0005.

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81-176 Cjp29      1 MEETREETPKTILSEWQEQFQNKETEIEKTLEKEKSSEELQKELEELNKL  50
CG8023 Cjp29      1 MEETREETSKTILSEWQEQFQDKETEIEKTLEKEKSSEELQKELEELNKL  50
                    ******** ************ ****************************

81-176 Cjp29     51 NFQEDLENQEVLKSQLEKQLKLEDLPQEAKQTLEILEEKEKLDNDMRNFI 100
CG8023 Cjp29     51 NFQEDLENQEVLKSQLEKELKLEDLPQEAKQTLEILEEKEKLDNDMRNFI 100
                    ******************.******************************

81-176 Cjp29    101 ELTTEYETPNEKRAFIENNLKEVRETPEINESLSILKEQDELQKEIDNTK 150
CG8023 Cjp29    101 ELTTEYETPNEKRAFIENNLKEVRETPEINESLSILKEQDELQKEIDNTK 150
                    **************************************************

81-176 Cjp29    151 EEMAREEKELKDQLENANKEKKGLSQEEYDEKFAEMEKEHAKKLKEMEEK 200
CG8023 Cjp29    151 EEMAREEKELKEQLE--NKEKKGLSQEEYDEKFAEMEKDHAQKLKEMEEK 198
                    **********.***  ********************.**.********

81-176 Cjp29    201 MEKLEQNFENSIENLLNSTSLSAILKSLYEMDKSLHLILKEKENNFDELN 250
CG8023 Cjp29    199 MEKLEQNFENSIENLLNSKSLSAILKSLYEMDKSLHLILKEKENNFDELN 248
                    ***************** ******************************

81-176 Cjp29    251 RQREEKLNLAMQTEKTREVVIDFIKELHQKTKGVENGISEMNKERNFYYE 300
CG8023 Cjp29    249 RQREEKLNLAMQTEKTREVVIDFIKELHQKTKGVENGISEMNKERNFYYE 298
                    **************************************************

81-176 Cjp29    301 LDKLTKNPEKMDLKETQKLQEYINYIDKETPNFKENYPKKYKQTKKIIEN 350
CG8023 Cjp29    299 LDKLTKNPEKMDLKETQKLQEYINYIDKETPNFKENYPKKYKQTKKIIEN 348
                    **************************************************

81-176 Cjp29    351 NIKKTMDKAKVKTANSQGQGMSL  373
CG8023 Cjp29    349 NIKKTMDKAKVKTANSQG--MSL* 370
                    ******************  ***
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FIG. 20. (A) Heterogeneity of electrophoretic mobility of Cjp29 amongst *C. jejuni* isolates. Whole cell lystates of *C. jejuni* 81-176 (Lane 1), *cjp29*::Cm (Lane 2), *C. jejuni* CG8023 (Lane 3), CG8023 *cjp29*::Cm (Lane 4) were immunoblotted and probed with polyclonal anti-Cjp29 antiserum at the indicated dilution (see Material and Methods). (B) Sequence alignment of *C. jejuni* 81-176 Cjp29 and *C. jejuni* CG8023 Cjp29.

Table 4. Strains used for Cjp29 analysis.

Strain	Description	Reference/source
81-176	Wild-type *C. jejuni* strain	Korlath et al., 1985
NCTC11168	Wild-type *C. jejuni* strain	Parkhill et al., 2000
CG8023	Wild-type *C.jejuni* strain	Tribble et al, in preparation
CG8071	Wild-type *C. jejuni* strain	Tribble et al, in preparation
CG8087	Wild-type *C. jejuni* strain	Tribble et al, in preparation
CG8261	Wild-type *C. jejuni* strain	Tribble et al, in preparation
PG1524	*C. jejuni* 81-176 pVir/*cjp29*::cat	Bacon et al., 2002
PG1874	*C. jejuni* 81-176 pVir/*cjp29*::cat +pAK101	This work
PG1777	*C. jejuni* NCTC11168+pVir/*cjp11*::cat	Bacon et al., 2002
PG2138	*C. jejuni* NCTC11168+pVir/*cjp29*::cat	This work
PG1709	*C. jejuni* 81-176 pVir/*cjp1*::cat	Bacon et al., 2002
PG1712	*C. jejuni* 81-176 pVir/*cjp2*::cat	Bacon et al., 2002
PG1411	*C. jejuni* 81-176 pVir/*cjp3*::aph3	Bacon et al., 2000
PG1409	*C. jejuni* 81-176 pVir/*cjp5*::aph3	Bacon et al., 2000
PG2101	*C. jejuni* 81-176 pVir/*cjp5*::aph3+pJl107	This work
PG1554	*C. jejuni* 81-176 pVir/*cjp6*::cat	Bacon et al., 2002
PG1731	*C. jejuni* 81-176 pVir/*cjp49*::cat	Bacon et al., 2002
PG1713	*C. jejuni* 81-176 pVir/*cjp53*::cat	Bacon et al., 2002
PG2177	*C. jejuni* CG8023 pVir/*cjp29*::cat	This work
PG2291	*E. coli* DH5α containing pJL104	This work
PG2292	*E. coli* DH5α containing pJL105	This work
DRH461	*C. jejuni* 81-176 Sm^R Δ*astA*	Hendrixson et al., 2003
PG2293	*C. jejuni* DRH461 containing pJL104	This work
PG2294	*C. jejuni* DRH461 containing pJL105	This work
PG2295	*C. jejuni* NCTC11168 containing pJL104	This work
PG2296	*C. jejuni* NCTC11168 containing pJL105	This work
PG2297	*C. jejuni* PG1777 containing pJL104	This work
PG2298	*C. jejuni* PG1777 containing pJL105	This work
PG2299	*C. jejuni* DRH461 pVir/*cjp2/virB9*::cat+pJL104	This work
PG2300	*C. jejuni* DRH461 pVir/*cjp2/virB9*::cat+pJL105	This work
PG2343	*C. jejuni* PG2138 (pJL104)	This work
PG2344	*C. jejuni* PG2138 (pJL105)	This work
DH5α	*E. coli* strain used for cloning	Gibco BRL
RK212.1	DH5α derivative used for conjugation into *C. jejuni*	Figurski 1979

CHAPTER 5

Discussion and Future Directions

Bacterial protein secretion systems have formed the basis for pathogenic schemes of a variety of animal and plant bacterial pathogens. A type of bacterial secretion system, designated type IV, has been shown to secrete DNA, protein, or nucleoprotein complexes across bacterial membranes (Christie, 2001). These secretion systems appear to be ancestrally related to conjugation machines, which similarly are capable of the translocation of DNA or DNA::protein complexes. The importance of type IV secretion systems (TFSS) to the pathogenesis of clinically relevant bacteria is resoundingly clear. *Legionella pneumophila* uses the Dot/Icm TFSS to subvert the organelle trafficking system of the macrophage in order to establish a replicative vacuole (Cascales and Christie, 2003). *Helicobacter pylori* uses the *cag* pathogenicity island TFSS to translocate the CagA protein into gastric epithelial cells, where it manipulates host cell signaling pathways (Odenbreit et al., 2000). Strains of *Neisseria gonorrhoeae* use a TFSS to secrete chromosomal DNA into the extracellular space, thereby aiding in the potentiation of genetic diversity (Dillard and Seifert, 2001). *Bordetella pertussis* secretes one of its major virulence determinants, the pertussis toxin, by a TFSS (Weiss et al., 1993).

Sequence analysis of one plasmid from *C. jejuni* 81-176 revealed the presence of eight homologs of a TFSS present in *H. pylori* and *W. succinogenes*. Mutational analysis of some of these genes suggested their involvement in intestinal epithelial cell invasion and natural competence (Bacon et al., 2000; 2002). Mutation of one of these genes, a VirB11 homolog, resulted in disease attenuation in the ferret diarrhea model, further suggesting a role for the pVir TFSS *in vivo* (Bacon et al., 2000). Although implicated in virulence, the exact contribution of the pVir TFSS remained

to be elucidated. Therefore, a broader characterization of the pVir TFSS was undertaken.

Upon the generation of antisera against VirB10, a structural component of the pVir TFSS secreton, it was observed that there was a discrepancy in mass between the recombinant form of the protein and the form expressed in *C. jejuni*. Since *C. jejuni* is known to post-translationally modify a number of its proteins, we investigated whether this protein was glycosylated and if this glycosylation affected the function of the TFSS.

Evidence suggests that the glycosylation of bacterial proteins may contribute to a variety of cellular and pathogenic processes. Reduction in bacterial adherence and invasion was demonstrated in several organisms when glycosylation was prevented (Benz and Schmidt 2001; Grass et al., 2003; Kuo et al., 1996; Lindenthal and Elsinghorst 2001; Moormann et al., 2002; Szymanski et al., 1999). Other work has implicated protein glycosylation in antigenic variation, protection from proteolytic cleavage, and solubility (Harris et al., 1987; Jennings et al., 1998; Kahler et al., 2001; Marceau et al., 1998). The flagella of *C. jejuni* 81-176 are extensively modified with *O*-linked pseudaminic acid residues and derivatives (Thibault et al., 2001). In the absence of any of these modifications, flagella filaments do not assemble, rendering the bacteria non-motile and thus non-virulent (Goon et al., 2003). Additionally, glycosylation deficient mutants in the *C. jejuni pgl* locus have a reduced capacity to invade INT407 cells and a deficiency in their ability to colonize the intestinal tracts of mice (Szymanski et al., 2002).

The findings reported here demonstrate that the *C. jejuni* 81-176 pVir TFSS protein, VirB10, is glycosylated at two sites, N32 and N97. Thus, the two forms of VirB10 observed in wild-type DB179, both of which bound to the SBA lectin column, represented mono- and di-glycosylated forms. The minor band seen in *E. coli* whole cells containing pJL101 (Figure 7, lane 3) most likely represents unprocessed VirB10. The small difference in mass (906 daltons) between the VirB10 signal peptide and the mass of the *N*-linked glycan (1406 daltons) was not resolved in SDS-PAGE. Glycosylation of VirB10 at N97, but not N32, was essential for wild-type levels of competence. The predominant modification site appears to be N97, since the unmodified form of VirB10 (40.5 kDa) was present in glycine extracts of the VirB10 N97A mutant, but not the VirB10 N32A mutant (Figure 9) the N32A site, perhaps due to increased surface exposure.

The original phenotype described for *pgl* mutants was loss of immunoreactivity with a variety of antisera made against *C. jejuni* (Szymanksi et al., 1999). This was interpreted as being due to the immunodominance of the glycan on proteins that were expressed at low levels. However, the glycosylated VirB10 protein appeared to lose reactivity in a *pgl* mutant background with antiserum generated against a recombinant, unglycosylated form of the same protein. This would suggest that in the absence of glycosylation VirB10 was either not transported to the periplasm or, upon transport, was unable to interact with the other components of the TFS apparatus and was rapidly degraded. However, the VirB10 N32A, N97A mutant protein was detected in glycine extracts when over-expressed *in trans* from the *flaA*σ^{28} promoter, which is approximately 10-fold stronger than the native *virB10* promoter (Guerry,

unpublished data). This would suggest that the lack of detection of VirB10 in the *pgl* mutants reflects instability of the non-glycosylated protein, perhaps a result of an inability to interact with other TFSS proteins.

The *A. tumefaciens* homolog of VirB10 has been previously shown to be an inner membrane protein that spans the periplasm and interacts with other TFSS components to form a functional secretion channel (Das and Xie, 2000). It is proposed in *A. tumefaciens* that VirB10 spans the periplasm in an oligomeric state and stabilizes interactions with other VirB proteins (Beaupre et al., 1997; Das and Xie 2000; Ward et al., 1990). In the absence of VirB10, substrates were not secreted, suggesting the secretion channel was not formed, underscoring the role of VirB10 in the functionality of the system (Berger and Christie, 1994). From computer prediction analysis, the *C. jejuni* VirB10 appears structurally similar to the *A. tumefaciens* VirB10 and is localized and functions similarly. Mutational analyses of the genes in the ComB system of *H. pylori*, which share homology with the pVir TFSS, resulted in severe reductions in natural competence, suggesting that these ComB proteins form a TFSS that is involved in DNA uptake (Hofreuter et al., 1998, 2001). In *C. jejuni* 81-176, mutation of *virB10* resulted in a modest effect on natural competence and a lesser effect on intestinal cell invasion (Bacon et al., 2000; 2002; Wiesner et al., 2003). The *pgl* mutants were previously shown to have a decreased capacity to adhere and invade INT407 cells (Szymanski et al., 2002), and in this study we have demonstrated that *pglB* and *pglE* mutants are severely reduced in natural competence, likely due, to a limited degree, to lack of VirB10 glycosylation. The greater competence defect exhibited in the *pgl* mutants when compared to the *virB10*

mutant suggests that additional glycoproteins are required for other steps in natural transformation. This notion is also consistent with the recent description of a putative type II secretion system involved in natural competence in *C. jejuni* (Wiesner et al., 2003). It is interesting that 8 of the 10 proteins described by Wiesner et al. contain putative *N*-linked glycosylation sites by computer prediction. It remains to be determined if any of these proteins are glycosylated and if their function will be affected if site-specific mutagenesis of their *N*-linked glycosylation sites is performed. Additionally, the observation that *C. jejuni* strains that lack pVir are competent also reinforces the notion that the pVir TFSS, while modulating competence levels, does not function as the primary DNA uptake system. Nevertheless, the modest effect of *virB10* mutation on natural competence has been demonstrated by two independent groups and was complemented *in trans*, suggesting the defect is genuine (Bacon et al., 2000 Wiesner et al., 2003, Larsen et al., 2004). Speculatively, mutation of *virB10* may have an indirect effect on natural competence by destabilizing other proteins that exist in the periplasm or membrane in the absence of a functional TFSS channel.

The identification of a TFSS structural protein that is glycosylated is significant on a number of levels. This is the first example, to our knowledge, of glycosylation of any TFSS protein, as well as the first function ascribed to an *N*-linked glycan in *C. jejuni* since glycosylation appears to affect the stability of VirB10. Secondly, the plasmid-encoded pVir TFSS was presumably acquired through horizontal transfer from an unknown donor. Interestingly, the closest homolog of *C. jejuni* VirB10 is found in *W. succinogenes*, which is also the only other bacterium known to contain a

putative *N*-linked glycosylation system homologous to the *C. jejuni pgl* system (Baar et al., 2003). Although the biochemical advantage of this general protein glycosylation system remains unknown, it would appear that the gene products of horizontally acquired DNA may be subject to functional constraints from the *pgl* glycosylation system and may need to be further modified to acclimate them to life within the *C. jejuni* host. The extrachromosomal nature of pVir suggests that the plasmid was acquired by horizontal transfer. The TFSS on pVir is thought to form a multi-protein complex and mutation of N97 on VirB10 appeared to affect stability of the protein and/or protein complexes in the periplasm. In the case of the pVir TFSS, it would suggest that VirB10 had to undergo a glycosylation mandated structural change in order for the protein to retain its functionality. Since *C. jejuni* is naturally competent organism, it is capable of constantly receiving DNA from a variety of organisms. This enforcement of post-translational conformity by a chromosomally encoded glycosylation system may be a mechanism of protection against unwanted or unusable genetic material.

Previous work has underscored the importance of the VirB11 family of ATPases in type IV secretion. In *H. pylori*, mutation of HP0525 resulted in an inability to translocate CagA into gastric epithelial cells (Fischer et al., 2001). Similar results were found by Krause et al. who demonstrated that mutation of the Walker A box of TrbB resulted in an inability to conjugatively transfer the RP4 plasmid (Krause et al., 2000). In *A. tumefaciens*, T-DNA transfer was shown to be dependent on the ATPase activity of VirB11 (Stephens et al., 1995). In this work, we first demonstrated the ATPase activity of Cjp5, a VirB11 homolog, was defined and

found to possess a weak *in vitro* ATPase activity, consistent with other VirB11-family members (Krause et al., 2000). Mutation of the consensus nucleotide-binding site (Walker A box) resulted in an enzymatically inactive form of Cjp5. A large amount of structural data on this family of proteins has been reported. Electron microscopy, gradient centrifugation, as well as X-ray crystallography have all provided evidence that the VirB11-family of proteins is capable of forming homohexameric ring structures (Krause et al., 2000; Yeo et al., 2000). In this study, through yeast 2-hybrid and chemical cross-linking of Cjp5, we provide preliminary evidence of a similar tertiary structure, suggesting that Cjp5 is another member of the VirB11 family of proteins. This work provides the first biochemical characterization of a pVir TFSS protein. This analysis provides confirmation of the *in silico* prediction of function for Cjp5/VirB11.

Although *C. jejuni* is generally considered an invasive organism, very little is understood about the molecular mechanisms of invasion. The identification of a plasmid-encoded TFSS implicated in intestinal epithelial cell invasion in the highly invasive strain 81-176 served as a preliminary explanation of the disparity among the invasion levels exhibited by various *C. jejuni* strains. One of the mutations identified in these studies, within *cjp29*, resulted in levels of intestinal epithelial cell invasion of about 15% of wild-type 81-176 (Bacon et al., 2002).

This work characterizes this protein, Cjp29, and shows that its expression is linked with the TFSS apparatus. Mutation of all eight TFSS genes and *cjp51*, a gene of unknown function, resulted in severely diminished levels of Cjp29 protein. This is the first example, to our knowledge, of such regulation within the TFSS family, and is

also a novel form of regulation for campylobacter species. These findings also break the association of the pVir TFSS with intestinal epithelial cell invasion. Since some mutants in the TFSS fail to produce appreciable amounts of Cjp29, but are fully invasive, the effects of mutation of *cjp29* on invasion remain unexplained despite the fact that the phenotype could be complemented *in trans*. This suggests that a high level of skepticism is warranted when analyzing putative invasion genes for an organism like *C. jejuni* that invades at levels that are lower than other enteric pathogens. This concern should be even greater for those strains of *C. jejuni* that invade at levels appreciably lower than 81-176, like NCTC11168 and F38011 (Konkel et al., 1999; Bacon et al., 2002). It may be that only more marked defects in invasion, such as those for a deep rough LOS mutant, which exhibit levels of invasion roughly 1000 times lower than wild-type (Kanipes and Guerry, in preparation), are biologically significant. This dissociation between invasion and the TFSS is also consistent with recent data that other clinical isolates containing pVir-like plasmids are non-invasive for INT407 cells *in vitro*, including the Thai strains reported here (Tribble et. al, in preparation).

Preliminary analysis suggests that *cjp29* expression is regulated at several levels. The first level of regulation appears to be repression that is mediated within the repeat region. This is based on the observation that deletion of the repeat region from pJL104, generating pJL105, was required to obtain detectable levels of AstA in the gene fusion experiments. Since detectable levels of Cjp29 protein were readily observed in Western blot when the gene was expressed by this same region of DNA in pAK101, it suggests that the *astA* fusion system is relatively insensitive compared

to immunoblot methods. Nonetheless, AstA activity could be detected from pJL105 lacking the repeats, and this higher level of expression was dependent on the TFSS encoded by pVir and by the presence of Cjp29 itself. Although there seems to be positive auto-regulation with Cjp29, the low pI of Cjp29 (4.67) makes it an unlikely candidate for a DNA binding protein. Therefore, there may be a third unidentified pVir-encoded protein that detects the presence of both the TFSS and Cjp29. In the absence of either component, *cjp29* expression would be down-regulated. It is currently unknown which pVir-encoded protein regulates Cjp29 or how this protein would sense the absence of a functional TFSS. It may be that *cjp29* expression is down-regulated *in vivo* until contact with a stimulatory signal is negotiated. Precedent for such an idea has been demonstrated with the *C. jejuni* Cia proteins, whose expression and secretion is induced by several factors, including bile salts and contact with cultured epithelial cells (Rivera-Amill et al., 1999, 2001).

Homologs of Cjp29 were present in 4 other clinical isolates from Thailand that were probe-positive for a TFSS gene. Sequence analyses of two of these *cjp29* alleles revealed that they encoded proteins with 97% identity to Cjp29 from 81-176. Cjp29 from 81-176 ran aberrantly in SDS-PAGE gels and this phenomenon was magnified further in the Thai isolates. Such heterogeneity in mass or electrophoretic mobility has been observed with proteins from other pathogens. For example, the CagF protein, encoded within the *cag* pathogenicity island of *H. pylori* (Censini et al., 1996), has been shown to vary significantly in its migration in SDS-PAGE gels (Seydel, et al., 2002), based on limited amino acid changes in different strains.

The homology of Cjp29 to multiple eukaryotic cell proteins, in addition to its coordinate regulation with the TFSS, suggests Cjp29 may be a secreted effector of the pVir TFSS. The observation that convalescent sera from individuals infected with *C. jejuni* reacts with a structural component of the TFSS suggests that the system is expressed during a human infection. This provides circumstantial evidence of the pathogenic involvement of the pVir TFSS in an *in vivo* setting. The level of similarity of Cjp29 to eukaryotic cell proteins is evidenced by the extensive cross-reactivity seen with antisera generated against recombinant protein expressed in *E. coli*. This cross-reactivity, while interesting, has hampered any meaningful analysis of the potential interaction of Cjp29 with eukaryotic cells by immunoblot analysis. Furthermore, attempts to express various tagged versions of Cjp29 in *C. jejuni* have not resulted in sufficient levels of expression for detection (data not shown). We cannot rule out the possibility that Cjp29 is not a secreted effector or that other cell types/systems would provide a more representative model for assessing the function of the pVir TFSS. Regardless, future attention should be directed to assessing the role of Cjp29 as it relates to the pVir TFSS and *C. jejuni* pathogenesis.

FUTURE DIRECTIONS

Although this work represents an important initial characterization of the pVir TFSS, much more work remains to define the specific contributions of this system to *C. jejuni* 81-176 pathogenesis. Although mutation of some of the structural components of the pVir TFSS resulted in decreased intestinal epithelial cell invasion *in vitro*, mutation of other components that are essential for function in other TFSS

had no apparent invasion effect. This observation would suggest that this system does not directly contribute to *in vitro* intestinal epithelial cell invasion or, more unlikely, components of the pVir TFSS are either redundant or unnecessary. Additionally, strains obtained from ill soldiers on exercise in Thailand contain plasmids that hybridize to a *virB11* probe and express Cjp29. These strains are noninvasive *in vitro* and vary in their clinical manifestations from watery diarrhea to a dysenteric-like illness, suggesting that the pVir TFSS does not directly mediate intestinal epithelial cell invasion (Guerry, Tribble unpublished data). Some of these isolates from Thailand clinically presented as bloody diarrhea but yet are noninvasive *in vitro*, suggesting that invasion *in vitro* may not correlate with dysenteric illness (Guerry, Tribble unpublished data). This work also suggests that the current criteria for determining invasion defects in *Campylobacter* strains may need to be refined. The decreases in *in vitro* invasion levels observed with the pVir mutants in strain 81-176 are most likely not biologically significant, since the decreases result in invasion levels that are still higher than other *C. jejuni* isolates (Bacon et al., 2000, 2002).

Most importantly, the field of *Campylobacter* needs development of *in vitro* and animal models that are representative and translatable to human infection. Everest et al., developed a differentiated Caco-2 model for *C. jejuni* invasion and was able to correlate clinical outcome with invasiveness in that model (Everest et al., 1992). However, some Thai strains, described above, which presented with dysentery in patients were noninvasive for Caco-2 cells (Guerry unpublished observations). Other nonpolarized cell lines, such as INT407, are useful for determining invasion level, but have not consistently corresponded to results in animal models (Guerry unpublished

observations). Currently, there are a number of useful non-surgical animal models for studying *C. jejuni*. The ferret model is useful for studying C. *jejuni* pathogenesis since it is a diarrhea model. However, in order for infection to result in disease the ferrets must be young and thus not fully immunocompetent. Additionally, the large infectious dose (10^{10}) makes the model not representative of infection in humans (Doig et al., 1996). Models such as the chicken and mouse are not useful for studying pathogenesis, since both are colonization models. There is a colostrum-deprived piglet model for diarrhea that does seem to correlate with clinical presentation in humans, but this model is difficult and costly and there have been few reports regarding its use (Babakhani et al., 1993). There is clearly a need for reproducible, inexpensive animal models that are representative of human infection.

The characterization of Cjp29 undertaken here provides some important clues to the function of the pVir TFSS. The observation of the linkage of Cjp29 expression with the pVir TFSS genes suggests that Cjp29 may be a secreted substrate of the pVir TFSS. Structural analysis using a variety of computer databases failed to idenitify any significant homology with known proteins. Attempts to demonstrate translocation of this protein into culture supernatant, INT407 cells, Caco-2 cells, and 28SC macrophages have been unsuccessful. Further work will be needed to develop optimal reagents to detect Cjp29 translocation, if it is in fact occurring. Secondly, it is currently not known if the cell lines used to examine Cjp29 translocation are a representative model. Again, the identification of a phenotype for the pVir TFSS will be essential for the establishment of such models. If translocation of Cjp29 into eukaryotic cells is described, a further analysis of the structural and energetic

requirements for TFSS substrate secretion could be elucidated. An example would be to use the VirB11 K156A mutant developed in this work to analyze the contribution of the ATPase activity of VirB11 to substrate secretion. Further, the VirB10 N97A mutant could be included in such analyses and would allow of the demonstration of a reliance on post-translational modification for function of the pVir TFSS.

If Cjp29 were found to be translocated into eukaryotic cells, an analysis of its function inside the cell could also reveal clues about the contribution of the pVir TFSS to pathogenesis. Screening of a eukaryotic cDNA library against Cjp29 using yeast 2-hybrid analysis could prove useful in determining the protein interaction partners of Cjp29 inside the eukaryotic cell. The identification of such interacting protein(s) would undoubtedly be useful in determining Cjp29 function.

Additional work could be undertaken to identify the plasmid-encoded proteins involved in the regulation of Cjp29. To date, there has been no report of any protein co-regulated with a TFSS. Additionally, this is a novel form of gene regulation in *C. jejuni*. The identification of the protein/s involved in this novel regulation could allow for significant advances in the study of the regulation of *Campylobacter* gene expression. Our current hypothesis of the involvement of a third, unidentified pVir-encoded protein involved in the Cjp29 and TFSS regulation could be tested using a bacterial 2-hybrid system and screening Cjp29 as bait against a pVir expression library. This would identify proteins capable of interacting with Cjp29 in *C. jejuni* and could identify the regulator.

This work represents an initial characterization of this TFSS. This body of work reveals an aspect of this TFSS that conforms to the paradigm of other TFSS found in other bacterial pathogens. Namely, biochemical characterization of Cjp5/VirB11 demonstrated that this protein is biochemically similar in structure and function to other VirB11 family members. Other characteristics of this system, however, do not fit into the existing paradigm of TFSS present in other pathogens. The presence of a glycosylated structural component, VirB10, within the pVir TFSS is unique, to date, to *C. jejuni*. This fact suggests that the pVir TFSS does impart some selective advantage because the strains that have obtained this system, presumably via horizontal transfer, had to post-translationally acclimate it to life inside of *C. jejuni*. To expend the energy necessary for such acclimation and to maintain the system, some selective advantage is probably imparted. Finally, the description of a protein, Cjp29, whose expression is dependent on the pVir TFSS is unique for both *C. jejuni* and TFSS. Both of these latter observations make aspects of the pVir TFSS unique compared to the other TFSS in bacterial pathogens.

Strains of *C. jejuni* vary markedly in their clinical manifestations and *in vitro* invasion levels. Such variations have led us to speculate that the phenotypic differences exhibited by *C. jejuni* 81-176, which causes a dysenteric-like illness and invades epithelial cells at levels much higher than other *C. jejuni* strains, are most likely due to genetic differences. The identification of a plasmid involved in the virulence of *C. jejuni* 81-176 containing homologs of a TFSS reinforced this notion. The determination of a function of the pVir TFSS may facilitate the understanding of the variation of clinical outcomes seen amongst various *C. jejuni* isolates. The

presence of similar plasmid encoded TFSS in non-invasive clinical isolates from Thailand suggests, along with other evidence, that this system does not directly contribute to intestinal epithelial cell invasion *in vitro*. The development of a dysenteric-like illness and the high levels of intestinal epithelial cell invasion that are observed with some *C. jejuni* isolates, in particular 81-176, are presumably due to some genetic determinants.

In order to determine an observable phenotype for the pVir TFSS, a plasmid cured derivative of 81-176 is needed. Attempts to cure pVir have been uniformly unsuccessful in our lab (Bacon et al., 2000, Larsen, data not shown). If a phenotype were established for the pVir TFSS, mutation of all components of this system should yield a similar defect as is observed with other TFSS. The electroporation of the pVir plasmid into NCTC11168 did not, however, enhance epithelial cell invasion, further underscoring the fact that the genes encoded on the pVir plasmid do not directly contribute to intestinal epithelial cell invasion. *C. jejuni* clinical isolates from Thailand that caused dysentery invaded intestinal epithelial cells *in vitro* at levels equivalent to or below *E. coli* K12. It is possible that intestinal epithelial cell invasion does not correlate with dysentery observed upon human infection with some *C. jejuni* isolates. However, this would contrast current models of pathogenesis of other, more invasive enteric pathogens such as *Shigella* and *Salmonella* species. It is also possible that the pVir TFSS contributes to immune evasion or the disruption of intestinal epithelial barrier function, both of which could potentiate the manifestation of dysentery. However, at this juncture, such statements are speculative. What is clear is that there is genetic and phenotypic heterogeneity among *C. jejuni* isolates.

Since the identification of virulence determinants has been extremely problematic for many *C. jejuni* investigators, it is the understanding of the nature and contribution of these interstrain differences as well as commonalities that will be paramount to understanding the pathogenesis of *C. jejuni*.

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